Systematic studies of *Heracleum* L. (Umbelliferae) and related genera in the Sino-Himalayan region

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Declaration

I hereby declare that the work contained within this thesis is my own, unless otherwise acknowledged and cited, and that this thesis is my own composition. This thesis has not in whole or part been previously presented for any other degree.

Jin-Hyub Paik

Plate 1. Field expedition in Gaoligongshan (Yunnan, China: 2005, 2006)
① Mountain peaks in Gaoligongshan ② Apiaceae species in Gaoligongshan ③ Filed pressing of plants ④ Mountain trail from Chukai to Stone gate ⑤ Natural habitat of *Heracleum forrestii* ⑥ Pressed *Heracleum* specimen ⑦ Du-long river

Abstract

The genus *Heracleum* (Apiaceae) includes 65-70 species, and is distributed across the Northern Hemisphere from North America to East Asia (Pimenov & Leonov, 1993). The Sino-Himalaya region (Nepal eastwards to SW China) is one of the most important distribution areas of *Heracleum* (with 10 species in the Himalaya and about 30 species in China). This thesis integrates a series of studies on the systematics of Sino-Himalayan *Heracleum* including allied taxa and a taxonomic revision of Nepalese *Heracleum* species.

The taxonomic value of the various morphological characters used in *Heracleum* is generally discussed and potentially important fruit morphological characters investigated alongside. Several characters were found to be distinctive, constant out of taxonomic importance. These included extent of development of the lateral wings, presence/absence of epidermal hairs, hair types, and the transverse sectional arrangement of the mericarp, including position of sclerenchyma of vascular bundles, rib development, pattern of endosperm, distribution of vittae and thickening of wing margins. Based on these characters, *Heracleum* was divided into at least 5 types that are also supported by molecular data (ITS DNA sequences).

In order to evaluate the monophyly of Sino-Himalayan *Heracleum*, and clarify the species relationships within this group, sequences of ITS, rps16 intron, and psbA-trnH spacer of *Heracleum* and related genera were studied. The resulting data matrix was analyzed using Maximum Parsimony and Bayesian analysis. Both ITS and rps16 intron regions show a good resolution of phylogenetic relationships down to species level. However, although psbA-trnH region has been successively tested in other plant families, it failed to resolve relationships among the major *Heracleum* clade. Only higher level resolution was successful. The results of our comparative phylogenetic study confirmed that the “Major *Heracleum* clade” (Sensu Downie) remains monophyletic. Within this clade at least six major lineages are defined: *Heracleum*, *Malabaila-Pastinaca*, *Zosima*, *Semenovia*, *Tordylium*, and *Tordyliopsis*. The Sino-Himalayan *Heracleum* lineage is seen to comprise three distinct sub-lineages (*Heracleum* group I, II and III) including several European *Heracleum* species. *Heracleum* is thus not monophyletic. Generic segregates of *Heracleum* proposed in past classifications,

such as *Tetrataenium,* can be correlated with these subclades. These phylogenies also helped resolve the taxonomic placement of some enigmatic taxa such as *Tordyliopsis brunonis* and *Heracleum millefolium*. These molecular data provide valuable characters for inferring relationships within the Sino-Himalayan *Heracleum*.

A taxonomic account of the species of *Heracleum* in Nepal is presented including a key to identification of the species is produced. A reinterpretation of the taxonomy of Sino-Himalaya *Heracleum* is presented based on these results including an account of the Nepalese species.

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Chapter 1 Summary of contents and aims

1.1 Overview

The genus *Heracleum* (Apiaceae) includes 65-70 species, and is distributed across the Northern Hemisphere from North America to East Asia (Pimenov & Leonov, 1993). One of the centres of diversity is China (29 species; Pu & Watson, 2005) and the other is the Caucasus (26 species; Mandenova, 1950). Plants of *Heracleum* are usually perennial or rarely biennial herbs, and are found in temperate forest regions of the northern hemisphere, particularly in mountain areas, but can also be adapted to wet or dry habitats. The taxonomy of *Heracleum* presents some complex and interesting problems because of the variety of morphological types, the lack of clear boundaries between many of the species and the confused nomenclature (Mandenova, 1950).

Heracleum has been extensively treated by several authors in many parts its range, giving rise to many infrageneric and infraspecific taxa, and conflicting classifications. These taxonomic differences of opinion are largely due to alternative interpretations on 'traditional' morphological and anatomic characters, and, apart from some preliminary studes, *Heracleum* has not yet received great attention from modern molecular research. However, recent high level molecular phylogenies have provisionally identified a well supported '*Heracleum* clade' within the Apioid superclade comprising: *Tetrataenium*, *Tordylium*, *Heracleum* and *Pastinaca*, and possibly *Conium*, *Malabaila* and *Zosima* (Downie *et al*., 2000a & b, 2001, Katz-Downie *et al*., 1999).

The Sino-Himalayan region is very important to the understanding of *Heracleum* and its related genera. The East Himalaya/SW China region is one of two centres of diversity of this widespread genus. Also, problems of morphological complexity and blurred generic boundaries in this region highlight its importance. the generic characters traditionally used to recognise *Heracleum* in the western part of its range do not work well in the Sino-Himalayan region. *Heracleum* species (e.g. *H. burmanicum*, *H. biternatum*) In SE Asia area (Tropical south India, Upper Burma, Thailand) have fruits that are quite unlike those of the Caucasian species, but the Sino-Himalayan species appears to provide a grade between these two areas (Hedge & Lamond, 1992). Consequently, the Sino-Himalayan species are crucial in a world understanding for *Heracleum* as it is here that the problems of generic delimitation are most acute.

Chapter four provides an overview of the morphology of *Heracleum* in the Sino-Himalayan floristic region and attempts to identify the most important characters for study of the genus in this area. Following a literature and specimen survey of the characters, Stem size (stout/slender), Cauline leaf sheath extent, fruit wing development (fruit wing vs body ratio), and dorsal and lateral rib distribution were found to be potentially useful for separating taxa. However, most of the species do not show singular distinguishing apomorphies, but rather a combination of several particular characters and character-states that have to be used to discriminate the species from each other.

In an attempt to identify further useful characters for taxon delimitation in the Sino-Himalayan *Heracelum*, chapter five presents an investigation of macro- and

micro-morphology. In this pilot study, leaf micro-morphology in Sino-Himalayan *Heracleum*, several epidermal characters (trichome type, the shape of epidermal cells, and the stomata characters) were seen to be of taxonomic importance in differentiating some species. In particular, *Tordyliopsis brunonis* (previously regarded as *Heracleum brunonis*) has a unique trichome structure with apical glands. *Heracelum obtusifolium* and *H. candicans* have long, ribbon-shaped trichomes that give a lanate texture to the abaxial surface of the leaves and *Heracleum franchetii* was the only species observed that had tetracytic stomata. Furthermore, these preliminary investigations suggest that there are some useful micro-morpholgoical characters that could be used for grouping species, but only the ribbon-shaped trichomes in *Heracelum obtusifolium* and *H. candicans* suggest relationships as synapomorphies.

The taxonomic value of the anatomical characters used in *Heracleum* are generally discussed and experimented (Chapter 6). Fruit anatomy appears to show more useful characters than vegetative characters and those of leaf micromorphology investigated in chapter 5. Allied genera such as *Pastinaca*, *Semenovia*, *Tordylium* and *Zosima* are well supported as distinct from *Heracleum* on the basis of these fruit characters. Fruit anatomy also provides characters to support the separation of the genus *Tetrataenium* from the remainder of *Heracleum*.

To find out the relationships among these groups further investigations were conducted into fruit surface micro-morphology (chapter 7). The results of these studies show that an examination of the micro-morphological characters of

mericarp using SEM, particularly trichome type and surface pattern of trichomes, is very useful to delimit generic groups. It can also give some potentially useful characters to indicate relationships within the component genera. The diversity of mericarp surface structure found here is surprisingly varied and much further work will have to be undertaken to consider variability and adaptive significances of the features of surface ornamentation.

In order to evaluate the monophyly of Sino-Himalayan *Heracleum*, and clarify the species relationships within this group, additional sequences of ITS, rps16 intron and psbA-trnH spacer of *Heracleum* and related genera were analysed (Chapter 8). Both ITS and rps16 intron regions show a good resolution of phylogenetic relationships down to species level. psbA-trnH spacer has confirmed the *Heracleum* clade but failed to resolve relationships among the *Heracleum* clade. The comparative analysis confirmed the monophyly and refined the circumscription of '*Heracleum* clade' *sensu* Downie. Within this clade several major lineages are defined: *Heracleum*, *Malabaila-Pastinaca*, *Zosima*, *Semenovia*, *Tordylium*, and *Tordyliopsis*.

Chapter 9 presents a synthesis of the results of this thesis, drawing together the findings of morphological and molecular investigations. Basing generic delimitations using monophyly as a fundamental criterion, we can begin to construct the following tentative classification for Sino-Himalayan *Heracleum* and allied taxa. Genera (*Heracleum, Pastinaca* (may include *Malabaila*), *Zosima, Tordyliopsis,Tetrataenium* (to include all Himalayan species, and possibly *Semenovia*) and *Heracleum millefolium* as a new genus

1.2 Aims of this study

This project integrates two major taxonomic research areas on the genus *Heracleum* and produces a taxonomic account of Nepalese *Heracleum*.

1.2.1 Morphology and anatomy

- To study the morphology of the taxa of *Heracleum* in the Himalaya and W China regions, searching for useful diagnostic characters and those that may be relevant in the classification.
- To look for potentially useful characters from anatomy and SEM study of fruits.

1.2.2 Molecular study

- To sequence informative genes to reconstruct the phylogeny in the genus *Heracleum*.
- To address the following main questions:

  I. Do the Asian representatives of *Heracleum* form a monophyletic group?

  II. What are the species relationships within the Asiatic group and with other species in *Heracleum*?

  III. What are the relationships with the allied genera *Semenovia* and *Tordyliopsis*?

  IV. What are the relationships with other genera included in the '*Heracleum* clade' delimited in previous studies?

  VI. What are the possible evolutionary relationships and biogeography in *Heracleum*, especially in the Sino-Himalaya region?

1.2.3 Taxonomic account of Nepalese *Heracleum*

- To produce a taxonomic account of the species of *Heracleum* in Nepal and present a key to identification of the species.
- To reinterpret the taxonomy of Nepalese *Heracleum* based on these result.

1.3 Value of the research

This Sino-Himalayan taxa are unusual in a worldwide context as many Asian *Heracleum* species have characters that degenerate, and generic limits, both within and with several other genera (e.g. *Angelica*, *Peucedanum* and *Semenovia*), are blurred. This work extends past molecular studies to include more Sino-Himalayan taxa and so contribute to the international effort to elucidate the phylogeny of this group of genera. As the Sino-Himalayan taxa are unusual in their diversity within the genus *Heracleum* then they are a crucial part of the story. Detailed studies of morphology and anatomy will be used to search for taxonomically informative characters to support the molecular groupings and contribute to revising the genus in this area. This study will result in a revision of *Heracleum* for the forthcoming Flora of Nepal. Patterns in these data will also contribute towards theories of biogeographic links between the Himalaya and wider Eastern Eurasia.

Chapter 2: General Introduction

2.1 Introduction

2.1.1 The family Apiaceae

The Apiaceae Lindley (Umbelliferae) is one of the most distinctive families of flowering plants, because of its characteristic inflorescences and fruit, and the chemistry reflected in the odour, flavour and toxicity of many of its members (Heywood, 1982). The Apiaceae includes many familiar edible plants [e.g. carrot (*Daucus carota* L.), parsnips (*Pastinaca sativa* L.), wild celery (*Apium graveolens* L.), fennel (*Foeniculum vulgare* Miller), dill (*Anethum graveolens* L.), and coriander (*Coriandrum sativum L.*)], as well as several highly toxic species [e.g. hemlock (*Conium maculatum* L.), water-Dropwort hemlock (*Oenanthe crocata* L.), and fool's-parsley (*Aethusa cynapium* L.)]. The family is composed of about 455 genera and 3600-3700 species and is cosmopolitan in distribution, but largely concentrated in temperate regions (Pimenov & Leonov, 1993). The Apiaceae can be divided into three subfamilies (Hydrocotyloideae, Saniculoideae, and Apioedeae) and 12 tribes (Drude, 1898). Existing familial treatments include those of Koch (1824), de Candolle (1830), Bentham (1867), Boissier (1872), Calestani (1905), Koso-Poljansky (1916), and Cerceau Larrival (1962), each of which are based largely on the morphology and anatomy of the fruit. Although there are known problems with convergent evolution of some of the fruit types, Drude's 1898 system remains the predominant system of classification. The most recent treatment of the family (Pimenov & Leonov, 1993) is merely an adaptation of the Drude's (1898) system.

The family is closely related to Araliaceae and the boundaries between these two families remains uncertain. Drude(1898) defined Hydorocotyloideae by their woody endocarps, the lack of free carpophores, and the absence of oil canals between the ribs of the fruit; however, have oil canals associated with the vascular bundles in the ribs (= companion canals) (Tseng,1967).

Hydrocotyloideae comprise 34 to 49 genera in two tribes (Hydrocotyleae and Mulineae) differing in fruit compression and the shape of the mericarp commissure. Because of this distinctiveness, Hydrocotyloideae have sometimes been recognized as a separate family (Hydrocotylaceae) (e.g. Hylander, 1945; Thorne, 1992), and members of the group have often been envised as intermediates between Apiaceae and the closely related family Araliaceae (Baumann, 1946; Cerceau-Larrival, 1962).

There is no single character which distinguishes the two families, though they can nevertheless nearly always be easily recognized (Tutin, 1980). Recent studies using molecular technique are demonstrating the problems with the traditional classification and are beginning to resolve generic and traditional affinities. For instance, 13/18 genera for which two or more sequences were included in a study by Downie *et al*. (2000b) were found not to be monophyletic (Downie *et al*., 2000b). However, among those problematic tribes, several molecular systematic investigations have confirmed the monophyly of Apioideae and its sister relationship to the monophyletic subfamily Saniculoideae (Downie & Katz-Downie, 1996; Downie *et al*. (1996, 1998, 2000a, 2000b); Katz-Downie *et al*., 1999; Plunkett *et al*., 1996b;). Within these studies, in consequence, a well supported '*Heracleum* clade' within the Apioideae was identified (Downie *et al*., 2000a & b,

2001, Katz-Downie *et al*., 1999).

2.1.2 Diversity of *Heracleum*

The genus *Heracleum* (Apiaceae) includes 65-70 species, and is distributed across the Northern Hemisphere from North America to East Asia (Pimenov & Leonov, 1993). One of the centres of diversity is China (29 species; Pu & Watson, 2005) and the other is the Caucasus (26 species; Mandenova, 1950). Within Europe eight native species are reported, and the most common species is *H. sphondylium* (Brummitt, 1968), a familiar plant of hedgerows and woodland margins. Plants of *Heracleum* are usually perennial or rarely biennial herbs, and are found in temperate forest regions of the northern hemisphere, particularly in mountain areas, but can also be adapted to wet or dry habitats (Fig. 2.1). An unusual characteristic of the genus is the wide variation in size, with some species barely reaching 15 cm (*H. minimum* Lam), and others growing up to 4 or 5 m high (e.g. *H. mantegazzianum* Sommer & Levier) ranking them among the tallest and largest herbs in Europe.

The taxonomy of *Heracleum* presents some complex and interesting problems because of the variety of morphological types, the lack of clear boundaries between many of the species and the confused nomenclature (Mandenova, 1950). The dorsally compressed flat fruits with distinctive clavate vittae (oil tube which is club-shape, gradually widening toward the apex or base in the carpel walls of the fruits of the Apiaceae), shorter than the length of the mericarp, are characteristic of many *Heracleum* species in the western parts of Eurasia (Brummitt, 1968).

However, for many Asian species this character degenerates and generic limits, both within and with several other genera (e.g. *Angelica*, *Peucedanum* and *Semenovia*), are blurred (Hedge & Lamond, 1992).

Past authors have attempted to revise *Heracleum* using morphological and anatomical data and several controversial classifications have been proposed adding to the nomenclatural tangle (Briquet, 1924; Kowal, 197; Mandenova, 1950) and giving rise to segregate genera and many infrageneric and infraspecific taxa. *Semenovia* is a Central Asiatic genus of 18 species, sometimes included within and sometimes separated from *Heracleum* (Pu & Watson, 2005; Watson, 1999). The monotypic Himalayan genus *Tordyliopsis* is also sometimes included within *Heracleum* (Pimenov *et al.,* 2000). However, the separation or inclusion of these segregate genera is problematic in many cases because these treatments did not take account of the high variation in characters used (Table 2.2). Additionally, good quality herbarium specimens and full documentation are lacking in many instances.

High level molecular phylogenies have provisionally identified a '*Heracleum* clade' within the Apioid superclade comprising: *Tetrataenium*, *Tordylium*, *Heracleum* and *Pastinaca*, and possibly *Conium*, *Malabaila* and *Zosima* (Katz-Downie *et al.*, 1999; Downie *et al.*, 2000a & b; Downie *et al.,* 2001). Among their '*Heracleum* clade' only *Heracleum* has representatives in the Sino-Himalayan region (Nepal eastwards to SW China), and the sampling within *Heracleum* has so far been rather limited. However, the Sino-Himalayan region is very important to the understanding of *Heracleum* and its related genera as there are ten *Heracleum*

species in the Himalaya, and about 30 species in China The East Himalaya/SW China region is one of two centres of diversity of this widespread genus, the Caucasus region being the other. Also, problems of morphological complexity and blurred generic boundaries in this region highlight its importance.

Very recently there have been a couple of major contributions to the study of *Heracleum* and related genera. A phylogenetic study on a range of Iranian Apiaceae within the Apioid super clade (Yousef *et al.* in press) has expanded sampling within the '*Heracleum* clade' to include some of the European members of *Heracleum*, *Semenovia*, and *Ainsworthia.* In this study the largest genus, *Heracleum* is not supported as monophyletic. The type of the genus, *H. sphondylium*, and its allies form a core *Heracleum* clade, albeit this group is not very well supported (Yousef *et al.* in press). Ten species of *Heracleum* occur in Iran, but only one Iranian species was included. This study also suggested that Chinese species *H. candicans* may represent a new genus and *H. pedatum* (previously *Vanasushava pedata*) was sister group to *Tetrataenium*. The next largest genus, *Pastinaca*, appeared as paraphyletic, with *Malabaila* nested within, a well supported clade that corresponds to Vallejo-Roman *et al*.'s (2006) *Pastinaca* group. Yousef *et al*. in their forthcoming paper (in press), request further study of this group.

A second study, but this time with a more West Asian focus (Logacheva *et al*. in press), has extended the sampling within SW Asia. This study represents all subdivisions of SW Asian *Heracleum*, as well as some representatives of the *Pastinaca* complex. In this study *Heracleum* was shown to be a polyphyletic genus, as its species fall into two different clades. One of these clades also includes the closely related West Asian Tordylleae members *Symphyloma* and

Fig. 2.1 The diversity of *Heracleum* species growing in the field.
1: *Heracleum sublineare* C. B. Clarke (East Nepal); **2**: *H. kingdonii* H. Wollf (SW China); **3**: *H. nepalense* D. Don (East Nepal); **4**: field collection of *H. nepalense*; **5-8**: *H. forrestii* H. Wolff (SW China). [**5-6**: *H. forrestii* growing high altitude, extreme conditions; **7-8**: *H. forrestii* growing open alpine meadows]. Scale bars: A= 1 cm; B, F, G, H = 10 cm; C = 20 cm.

Mandenova. Although in general there was a good concordance with Mandenova's system of *Heracleum*, sections *Villosa*, *Apiifolia* and *Pubescentia* were not fully confirmed.

Both of these studies have confirmed the earlier results and suggested that *Heracleum* is polyphyletic and there is no good concordance with previous classifications. The present research is independent of these studies, but extends the sampling into the Himalaya and China.

2.1.3 Sexual system, Pollination and Hybridization

Reproduction is the most important event in a plant's life cycle (Crawley, 1997). *Heracleum* reproduces only by seed and reproduction by apomixis or vegetative means has never been observed (Perglová *et al*., 2007). As in other Apiaceae, *Heracleum* has unspecialized flowers, which are promiscuously pollinated by unspecialized pollinators, Many small, closely spaced flowers with exposed nectar makes each insect visitor to the inflorescence a potential and probable pollinator (Bell, 1971). A list of insect taxa sampled on *H. mantegazzianum* (Grace & Nelson, 1981) shows that *Coleoptera*, *Diptera*, *Hemiptera* and *Hymenoptera* are the most frequent visitors.

Heracleum has an andro-monoecious (andro-polygamous: having staminate and perfect flowers on the same plant) sex habit, as has almost half of British Apiaceae (Lovett-Doust & Lovett-Doust, 1982). Together with perfect (hermaphrodite) flowers, umbels bear a variable proportion of male (staminate) flowers. The species are considered to be self-compatible, which is a typical

feature of Apiaceae (Bell, 1971), and protandrous (Grace & Nelson, 1981; Pergová *et al*., 2006). Protandry is a temporal separation of male and female flowering phases, when stigmas become receptive after the dehiscence of anthers to prevent self-fertilization. It is common in umbellifers. Although protandry has traditionally been considered to be a mechanism of avoiding or reducing selfing, it is unlikely to guarantee outcrossing. However, when it is strongly developed, the male and female phases of a plant may be completely separated in time so that outcrossing is assured (Webb, 1981; Snow & Grove, 1995).

Umbels of different orders flower in sequence. The terminal (Primary) umbel is the first to flower, followed by secondary, and later tertiary and quaternary umbels on satellite and branches (Pergová *et al*., 2007). Flowering of *Heracleum* have a distinct architecture. The inflorescences are compound umbels of four orders. The main flowering shoot develops as a leafy stem that terminates in a primary (first-order) umbel, also called ‘terminal’, Lateral shoots, which are produced on the stem, terminate in secondary (second-order) umbels and can be found in a satellite position, surrounding the primary umbel. Third-order umbels may arise on the shoots branching from secondary shoots and fourth-order umbels on shoots branching from tertiary shoots. Under favourite conditions, strong plants can produce several other shoots, which arise from the base of the flowering stem at ground level (basal branches). The character of the terminal umbles of these basal branches varies and in terms of umbel size, fruit size, fecundity and proportion of male flowers in intermediate between typical first and second-order umbels (Pergová *et al*., 2007). Each compound umbel consists of umbellues (umbellets), simple umbels that bear a large number of small, closely packed

flowers. Flowers are either hermaphrodite or male (staminate). The latter usually contain reduced stylopodia (Bell, 1971) and no, or a shrunken, style. The proportion of male flowers increases in higher-order umbels, while the terminal umbel usually contains only hermaphrodite flowers. If present, male flowers are located in the central part of umbellues. Within the same umbel, the proportion of male flowers seems to be the same in all umbellues (Perglova *et al*., 2006). Fourth-order umbels usually consist only of male flowers.

A study of flowering phenology conducted on plants growing in the experimental garden revealed that the overlap in anther dehiscence and stigma receptivity in flowers of the same umbellet. Such overlaps only occur in some umbels and only a small proportion of the late dehiscing anthers are usually involved (Perglova *et al*., 2006). Consequently, stigma are not covered by a mass of pollen from the same umbel but geitonogamous selfing (pollination between flowers of the same plant) can occur. Self-pollination was identified as advantageous in some colonizing species (Brown & Burdon, 1987) and selfing may lead to acceleration of the rates of spread (Lewis, 1973; Daehler, 1998). Of the all introduced species include in a study of Western Australian members of Apiaceae (Keighery, 1982), all the naturalized species were capable of autogamy and self-fertile. They all possessed attractive inflorescences, and were pollinated by a variety of native and introduced insects.

The ability to self is advantageous for successful colonization following long-distance dispersal of a single propagule, because there is no need to wait for a sexual partner (Baker's law; Baker, 1955). In early stages of invasion, when populations are small, plants should self to maximize fertility. Later, when populations are large and pollinators and/or mates are not limiting, outcrossing is

more beneficial because it generates increased genetic polymorphism (Pannell & Barrett, 1998; Rejmánek *et al*., 2005).

Heracleum mantegazzianum is reported as fully self-compatible, selfed fruit set was not lower than that of naturally pollinated flowers, and it does not suffer from inbreeding depression at the germination stage. The study of flowering phenology of *H. mantegazzianum* (Perglova *et al*., 2007) showed that overlaps between male and female flowering phases allow for geitonogamous pollination. This indicates that plants of *H. mategazzianum* are probably highly self-fertile if isolated or growing in very sparse populations where pollinators transport pollen within a single plant.

Interspecific hybrids between *H. mantegazzianum* and *H. sphondylium* are reported from Great Britain (McClintock, 1975) and Germany (Ochsmann, 1996). Hybrids are found in sites where both species grow together, although they are not numerous (Grace & Nelson, 1981; Stewart & Grace, 1984). This was studied and it was found that only experimental crosses in which *H. sphondylium* was the female parents were successful (Stewart & Grace, 1984). In this experimental cross only a negligible fruit set (1%) was obtained when pollen was transferred between flowers within the terminal umbel, which is consistent with rare overlap in anther dehiscence and female receptivity in the same umbel. However, they were able to realize high levels of selfing (68% fruit set) in two parents with incomplete separation of staminate and pistillate phases between umbels.

There has been no report of Hybrids between two species from Sino-Himalayan area yet. However, problematic delimitation and morphological complexities of many species are considered due to hybridization events (Watson, 1999).

By providing a test that is relatively independent of morphological classifications, DNA sequence data may help to understand the systematics of this genus. Investigating independent genomes (e.g., nuclear and chloroplast) facilitates recognition of patterns and processes such as hybridization events (e.g. Francisco-Ortega *et al*., 1996; Doyle & Doyle, 1998). Chapter 8 of this thesis provides DNA sequence data (nuclear: ITS, chloroplast: rps16 and psbA-trnH) and shows that there is no obvious conflict between nuclear tree and chloroplast tree. These results indicate that there are no obvious recent hybridization events among the taxa sampled for this experiment. However, a relatively limited number of accessions could result in such events being missed in the genus. Therefore, more extensive sampling is required and for a thorough analysis of hybridization status the genus.

2.1. 4 Economic uses

Heracleum has a very long history of use by people dating back to the earliest civilisations. Today it is important as a source of medicines, food, and fodder. Some species are of ornamental value, but others are problematic weeds. *Heracleum mantegazzianum* is notable for its importance in ecological research as in the UK it is an invasive alien and a public health hazard due to the phototoxicity of the sap (Jahodová *et al*., 2007)

1. Food and Fodder

Several species of *Heracleum* are utilized in rural traditions as human food or cattle fodder. In the Himalayan regions many *Heracleum* species (e.g. *H.*

candicans, *H. nepalense*, *H. wallichii*, *H. cachemericum*) are particularly useful for feeding goats or dried and stored to be given to livestock during the winter season (Pandey, 2000). In Europe, after World War II, Giant Hogweed (*H mantegazzianum* - under the name of *H. sosnowskyi*) was tested and used forage (silage) plants in the USSR and the results of the work on the introduction of Hogweeds as forage plants are summarized by Satzyperova (1984).

A few species are also used as vegetables for human consumption, and the fruit are consumed as a spice or flavouring agent and other various uses of the parts of the *Heracleum* are also found in rural traditions in some Himalayan countries (e.g. Pandey, 2000). In Northern and North-eastern China, young parts of *H. moellendorfii* (Da-ye-qin: Large-leaved Celery; Lao-shan-qin: Old Moundtain Celery) are used as a salad or vegetable (Hu, 2005).

2. Medicine

In Europe, *Heracleum* species have been used as important medicial plants from the early civilisations. Among them *Heracleum sphondylium* is most common. One of the finest early works recording this use is *De Materia Medica* (Pedanius Dioscorides ca. 64CE, from English translation of Osbaldeston and Wood, 2000) which records:

• The seed of this (taken in a drink) purges phlegmy stuff through the bowels. Taken in a drink it cures the liver, jaundice, asthma, epilepsy and constriction of the womb. Inhaled, it revives those who fall in a faint. If the head is moistened with it (with oil), it is good for fever of the brain, lethargy, and headaches. Applied

with rue [3-52] it restrains *herpes* [viral skin infection]. The root is given to the jaundiced and liverish. Shaved and inserted it eliminates the hardness of fistulas [ulcers]. The fresh juice from the flower is good for ulcerated and purulent ears. It is also preserved, placed in the sun like other juice. It is also called *arangem*, *phalangium*, *asterium*, *nisyris*, *sphondulis*, *chorandanon*, or *oenanthe*; the Romans call it *herba rotularis*, the Egyptians, *apsapher*, and the Magi, *osiris*.

In Sino-Himalaya region, many of the *Heracleum* species also have been used as important traditional medicine. Among them *Heracleum candicans* is popular and known as “Gandhrain” or “Gandharyan”. The raw root is considered a potent aromatic, stimulate, carminative, anti-colic, digestive and nervine tonic and seeds are orally given in condition of abdominal colic and other troubles of digestion and flatulence (Pandey, 2000).

In addition to medicinal uses in human beings, the plant is also considered useful as veterinary herbal medicine. The plant is suggested to be useful in conditions of abdomal disease, bone troubles, diarrhoea, indigestion and also general tonic for livestocks. (Pandey, 2000)

3. Invasive weed and public health hazard to humans

The genus *Heracleum* includes ecologically important and conspicuous species, but some are a health hazard to the public. Giant Hogweed, *Heracleum mantegazzianum,* is native to the sub-alpine and alpine belt of the Western Greater Caucasus. In the early 19th century was introduced to Western Europe as an ornamental plant, and prized for its towering statuesque form (Ochsmann, 1996). However, it readily sets copious, wind-blown seed and within 150 years it

had naturalized along waterways and roads and on fallow and disturbed land all over Europe (Pyšek *et al*., 2007). Good competitive ability and high seed production (Perglová *et al*., 2007), combine to make it an aggressive invasive weed causing problems in many habitats (Otte and Franke, 1998; Weber, 2004). Because the species possesses a broad array of chemical (furanocoumarins) and a good mechanical defence mechanism (a dense covering of stiff trichomes) it has few preditors. Where it grows as an invader it can form dense stands to the exclusion of other species and so reduce the biodiversity of structure and function within these ecosystems. The plant has a strong resinous smell and can cause serious UV-induced photodermatitis when it is handled in bright sunlight. Because the plant sap has furanocoumarins, it is a hazard to the public (Lagey *et al*., 1995). Wild parsnip (*Pastinaca sativa*) can also cause phytodermatitis too. Blisters form in a few hours after scrubbing parsnip stem onto the back of the hand under exposure of sunlight (personal experiment). This fits with the close relationship shown in molecular studies as well (see chapter 8).

2.2 Taxonomic history

2.2.1 The origin of the name

The origin of the name of *Heracleum* is still unclear, but the first known use was by the Roman naturalist Pliny (23-79 AD) who used it in memory of the Greek hero Heracles (Elder, 1634; Mandenova, 1950). Theophrastus (ca 370-285 BC) and Dioscorides (ca. 40-90 BC), Greek contemporaries of Pliny, used the name as an adjective '*Panax Heracleon*'. What this plant name actually refers to is still a matter of debate. Early workers such as Thellung (1925-6) and Gunther (1959) thought that Dioscorides used Panax Heracleon to refer to *Opopanax hispidum* or *Ferula opopanax*. However, recently, Osbaldeston & Wood (2000) threw doubt on this and insead considered that Dioscorides may have used it to refer to a species of *Heracleum* (such as *H. panaces, H. sphondylium, H. gumminifera, H. pyrenach*). This is based on the use of '*Panaces Heracleon',* '*Panaces Heraclium'* or '*Panax Heracleon'* by Caesalpino (1583), Dodoëns(1583) and a number of other authors (Bauhin, 1623; Ray,1686) to refer to various European members of the genus *Heracleum*. However, the early use of this name is still obscure. It is equally unclear as to which plant Dioscorides used the name *Sphondylium*. According to Thellung (1925-6) the plant described by Dioscorides is most likely to be '*Pastinaca sativa'*. But according to Gunther (1959) and Osbaldeston & Wood (2000) the plant is '*Heracleum sphondylium'*. Caesalpino (1583) and Dodoëns(1583) and a number of other authors used the name '*Panax Heracleon'* and '*Sphondylium*' separately in their books, but later Bauhin (1623), Morison (1672), Tournefort (1694), Ray (1686), Magnol (1720), Boërhaave (1725), and Royen (1740) all used the name *Sphondylium* in the contemporary sense of

Heracleum.

It was Linneaus who settled on *Heracleum* as the name for the genus (Fig. 2.2). In the first edition of *Systema Natureae* (1735) Linneaus first mentioned the species he called '*Heracleum sphondylium'*, as a species growing in the Western Europe. Later, Linneaus included five species within the genus *Heracleum* in his *Species Plantarum* (1753).

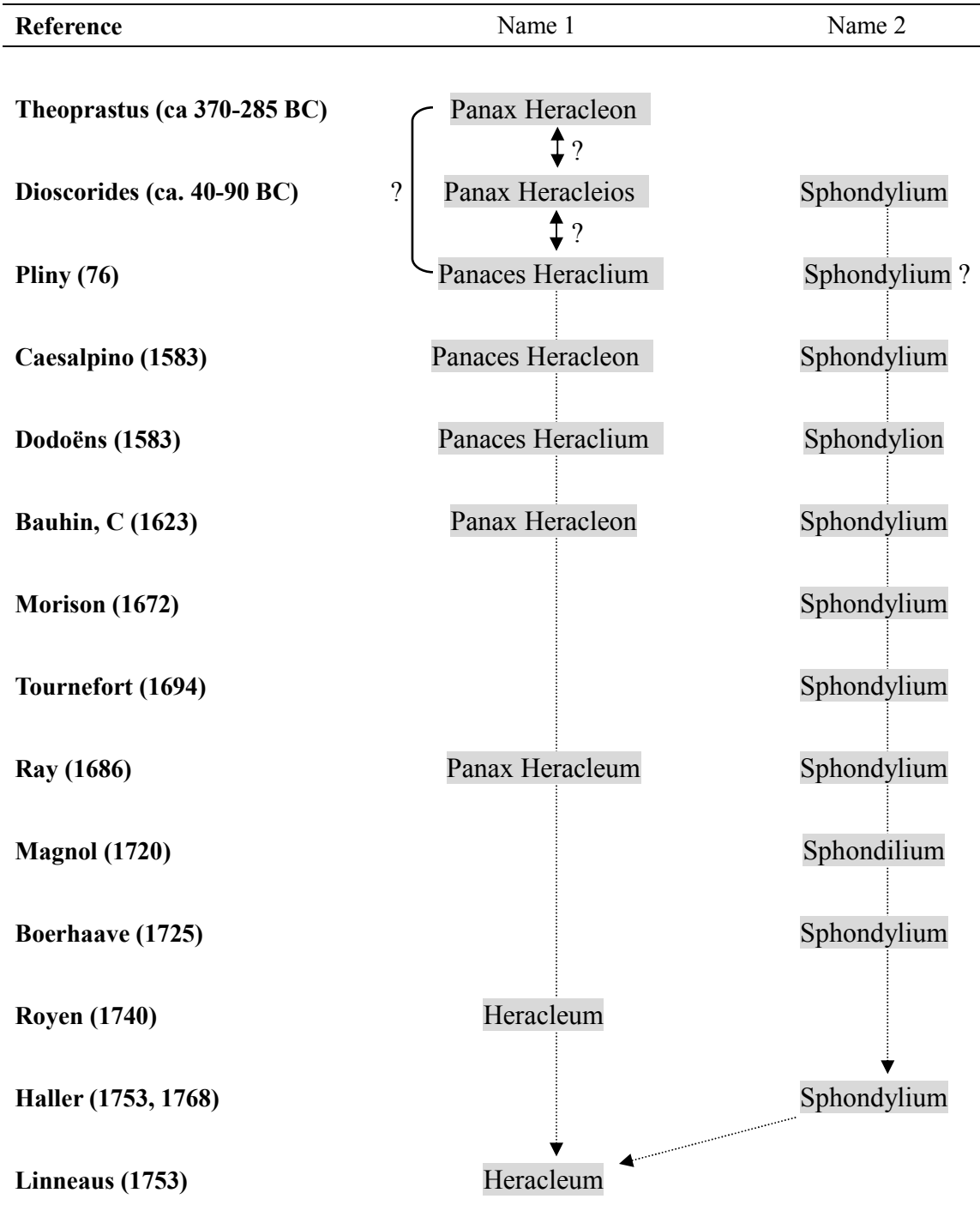


Fig. 2.2. The diagram of pre-Linnean historical name trace of *Heracleum*

2.2.2 Generic history of *Heracleum*

The generic delimitation of *Heracleum*, has long been controversial. As discussed above, it was Linnaeus who first recognized the genus. He included it, in his sexual system in *Systema Naturae* (1735) and *Species Plantarum* (1753): classified in Class V: Pentandria, Order Digynia. In *Species Plantarum* Linnaeus included five species in his concept of *Heracleum*: *Hercleum sphondylium*, *H. sibiricum*, *H. panaces*, *H. austriacum*, and *H. alpinum*. These species were distinguished from each other by leaf form, leaf shape and petal shape. Linnaeus was familiar with very few species of *Heracleum* and mainly the European ones, so all of these names are still recognized as a good species today.

Hoffmann (1816) limited the genus *Heracleum* to *H. sibiricum*, a species characterised by its greenish-yellow flowers and the almost un-enlarged, non-radiate petals of its marginal flowers. *Heracleum sphondylium* has white flowers and significantly enlarged radiate outer petals, and he transferred this to a separate new genus *Sphondylium*, and renaming it as *Sphondylium branca ursina*. He also included within *Sphondylium* a number of new species cultivated in the Gorenkov gardens that originated from the Crimea and the Caucasus (Mandenova, 1950). In order to distinguish the two genera by something other than the colour of their flowers, Hoffmann suggested further distinguishing features: in particular the shape of the small oil channels “vittae” in the walls of the fruit. Thus, the genus *Heracleum* (in Hoffmann’s sense) is characterized by narrow commissural vittae, with an insignificant degree of widening into a claviform shape at the lower end only. He also first described the genus ‘*Wendia*’ and compared it as an ally of his *Sphondylium*. There is still considerable

divergence of opinion among taxonomists about the generic independence of *Wendia*.

Berchtold and Presl (1820) proposed the family classification for the Apiaceae with eleven tribes, and included *Heracleum* within Tribe Selineae. However, they provided little evidence for the basis of their classification and it is unknown which characteristics they used to establish thier genus concept.

In 1830 De Candolle also included the genus *Heracleum* in his *Prodromus Systematis Naturalis*. De Candolle divided *Heracleum* into six sections on the basis of vittae number, flower shape and petal colour. He described the genus *Tordyliopsis* to include his new species (*T. brunonis*) on the basis of herbarium specimens recently collected in Nepal.

In 1866 Regel & Herder described the genus *Semenovia* for a new species based on herbarium specimens collected by P. P. Semenov in the Zailisky Altai mountains. Although this genus is very similar to *Heracleum* species that grow in this area, the authors chose to compare their new genus with *Pastinaca* and *Orlaya*. Perhaps this inappropriate comparison is the reason why this genus was not mentioned at all in later large syntheses such as Bentham & Hooker (1867) and Later O. & B. Fedtcheko (1911) included this plant in the genus *Heracleum* (Mandenova, 1950).

Bentham (1867) refined the delimitation of *Heracleum* by using several characters (petal shape, fruit margin shape, vittae number etc.), and he treated

Tordyliopsis within *Heracleum.* It was just over 100 years since Linnaeus first published the genus and in the century 70 species had been described that were now included in *Heracleum*. Bentham did not undertake any revision of the species within *Heracleum* , and even though *Heracleum* had become rather a large genus, no infra-generic classification was proposed.

In 1839 Bunge published a description of a new genus *Barysoma.* But there have been disagreements about the diagnosis of the genus *Barysoma* (Bentham & Hooker, 1867; Boissier, 1872). Bunge suggested the species *H. villosum* Hoffm. as a possible synonym, but the origin of the plant described by Bunge as *Barysoma villosa* is sadly unknown to us. In later works *Barysoma* has not been accepted, but treated as a synonym of the genus *Heracleum* (Mandenova, 1950).

Drude (1898) intensively investigated the occurrence of calcium oxalate crystals in the pericarp throughout the family and published an extensive treatment of Apiaceae that has largely stood the test of time and underpins our current understanding of the family. He delimited the three subfamilies: Hydrocotyloideae, Saniculoideae and Apioideae, and revised the infra familiar classification and delimitation of many genera. He characterized *Heracleum* on stylopodium shape, wing shape and clavate vittae size. He did not accept many of the past generic segregates of earlier workers and included *Tordyliopsis*, *Wendia*, and *Barysoma* within *Heracleum*, and placed the genus within Tribe Peucedaneae in subfamily Apioideae.

Calestani (1905) based his rather radical reclassification of the Apiaceae almost entirely on anatomical structure of the fruit, and significantly enlarged many genera. Thus, *Pastinaca* as visualised by Calestani includes a whole group of undoubtedly independent, though closely related genera, such as *Pastinaca* s. str., *Malabaila*, *Heracleum*, *Zosima*, and *Tordylium*. The view of Calestani was later developed by Koso-Poljansky (1916) and he accepted the genus *Pastinaca* as circumscribed in the work of Calestani (Mandenonva, 1950).

Koso-Poljansky (1916) extened Calestani's classification and proposed his multi-rank classification of the family almost entirely on fruit anatomical characters. Koso-Poljansky has the following divisions: *Branca-Ursina* K. -Pol. (= *Heracleum s. l.*) separated into *Heracleum* Calest., *Tordylium* Calest. and *Condylocarpus* K.-Pol. In its turn *Heracleum* Calest. (=*Heracelum* L.), is sub-divided into two groups: A. *Sphondylium* K.-Pol. (=*Sphondylium* Hoffm.; *Trigonosciadium*, Boiss.) B. *Wendida* K.-Pol. (=*Wendia*, Hoffm., *Wendia* auct). This radical and controversial reclassification of the family and delimitation of the genera was never widely adopted and remains an interesting but mostly ignored system.

Briquet (1924) carried out a detailed anatomical study of the fruit of most European and many near-Asian species of *Heracleum*. One of the purposes of his research was to investigate the possible further use of anatomical data for revising the taxonomy of *Heracleum*. However, this attempt was unsuccessful because it turned out that all the species he studied had a more or less homogenous pericarp structure. On the basis of anatomical study of the fruit of the various species of the genus *Heracleum*, Briquet came to the conclusion that

the obsolete feature of vittae on the commissure of the fruit had no taxonomic significance, and therefore *Wendia* could not be maintained as an independent genus, and even not as a section within *Heracleum*. Briquet did elucidate the taxonomic position of the enigmatic *H. pastinaca* Fenzl, which Boissier had separated into the section *Pseudotragium*.

Cerceau-Larrival (1962) developed a second rather radical reclassification of the family, this time largely based on the correlations between pollen morphology and the presence or absence of either round or long cotyledons. This was supported by evidence from inflorescences, fruits, and adult vegetative morphology. She grouped the 83 sampled genera into 38 tribes, and informally indicated a new tribe Heracleae to include only *Heracleum*, defined on the combination of characters of fruit morphology, pollen and cotyledon shape. Unfortunately, she did not clearly indicate which species she was including within her delimitation of *Heraleum*, and as sampling across the whole family was limited, her generic groups have largely been a source of taxonomic interest rather than a basis for a reclassification.

2.2.3 Infra-generic history of *Heracleum* (Table 2.1)

Infra-generic delimitation within *Heracleum* is as controversial and varied as the taxonomy at and above the generic level. **Koch (1824)** attempted a classification of the species within *Heracleum* for the first time. He used some of the features used previously by Hoffmann (1816) for the genera *Heracleum*, *Sphondylium*, and *Wendia* to produce an infra-generic grouping of the European species. In his book *Plantarum Umbelliferarm* he recognized 13 tribes within the family using basic fruit anatomical characters, and he used morphological characteristics of the petals to distinguish different genera. In his generic concept, *Heracleum* was comprised of eight species, which were subdivided by vittae number and flower shape

***Sphondylium* Hoffm**. (Commissural face 2-vittae, flowers radiate)
1. *H. sphondylium,* 2*. H. longifolium,* 3. *H. pyrenaicum,* 4. *H. villosum,*
5*. H. ligusticifolium*
***Heracleum* Hoffm**. (Commissural face 2-vittae, flowers sub-equal)
6. *H. sibiricum*
***Wendia* Hoffm**. (Commissural face 0-vittae)
7. *H. alpinum*, 8. *H.austriacum*.

J. E. Duby (1828) was the first to reduce Hofmann's genus *Wendia* into section Wendia within *Heracleum*, and following Koch, he included within this section the species *H. alpinum*, growing in the European Alps. Duby also regarded the genus *Sphondylium* Hoffm. as only a section *Sphondylium* (Hoffm.) Duby, to which he added *H. minimum* Lam.

After exploration was progressing fast, the most important early contribution to the understanding of *Heracleum* was made by **De Candolle (1830)**, who divided the

26 species of *Heracleum* known at that time into 6 sections: Sect. *Tetrataenium*, Sect. *Euheracleum*, Sect. *Sphondylium*, Sect. *Carmelia*, Sect. *Wendia*, and Sect. *Trichogonium*. De Candolle gave paramount significance to the colour of the flowers and the number of channels on the fruit commissure, and created a very artificial classification system for the genus. De Candolle's system often treated species that we now interpret as closely related in quite distant groups and also species with only tenuous or inconsistent ties were brought together. For example, De Candolle included the Nepalese species in a special Section *Trichogonium*, however, commissural vittae are not clearly visible and in the totality of its features *H. wallichii* is closer to other Nepalese species *H. nepalense* and *H. obtusifolium*. These latter species have four vittae on the commisure and De Candolle included them within Section *Tetrataenium*.

Koch (1837) followed De Candolle's division of *Heracleum* into sections, but broadened Section *Euheracleum*, by including Section *Sphondylium* within it.

Later **Ledebour (1844-46)** also followed De Candole and Koch's classification, and he placed the species growing in the territory included by "Flora rossica" into two sections: Sect. *Euheracleum* and Sect. *Wendia*. However, by this time, with the increasing number of known species of *Heracleum*, scientists were faced with the increasingly obvious artificiality of the sectional divisions of the genus proposed by De Candolle (1830).

In "Flora Orientalis" **Boissier (1872)** included the 27 species known to him in the Orient, but did not accept De Candolle's division. Instead, Boissier divided

Heracleum into two sections based primarily on the development of bracteoles: Sect. 1 *Euheracleum* – Umbellae involucellatae, and Sect. 2 *Pseudotragium* – Involucre et involucella nulla. In Section *Euheracleum* Boissier included species with yellow and white flowers, and species with well developed vittae on the commissure. Section *Pseudotragium* was created for a single species – *H. pastinaca*, growing in the Taurus Mountains. The species *H. carmeli*, which De Candolle had placed in a special Section *Carmelia*, was separated by Boissier into a new monotypic genus *Synelcosciadium*. Boissier further grouped the species within Sect. *Euheracleum* on the basis of the segmentation of the leaf blade.

C.B. Clarke (1879) chose not to divide the Indian species of *Heracleum* into Sections. Instead he artificially and informally grouped the Indo-Himalayan species known to him on the basis of the colour of the fruit and its pubescence. As in other genera in Flora of British India, these groupings were to aid identification and not indicate taxonomic affinities.

Drude (1898), in his seminal treatment of the family, observed that he did not have as yet sufficient information to divide *Heracleum* into Sections, and so he too avoided any sectional division of the genus. Instead, he placed all the sections established by De Candolle in the synonymy of the genus *Heracleum*.

Mandenova (1950, 1962, and 1986), mainly based on the Caucasian *Heracleum*, introduced a classification of *Heracleum* into sections: *Heracleum* L., *Villosa* Manden., *Pubescentia* Manden., *Apiifolia* Manden., *Wendia* (Hoffm.) Manden.,

Vocontia (Calest.) Thell., *Lasiopetala* Manden. *Pseudotragium* Boiss. This division are mostly based on fruit outer morphological characters, such as shape and length of dorsal vittae and present or absence of commissural vittae.

Tamamschjan (1967) in her account of the Caucasian *Heracleum* adopted only three sections – *Heracleum* (including Mandenova's *Villosa* and *Pubescentia*), *Apiifolia* Manden. and *Wendia* DC.

Satzyperova (1984), followed Mandenova's system in general, however, she proposed some subsections Subsect. *Sibirica* Satzyperova, Subsect. *Pontica* Satzyperova, *Sphondylia* (Manden.) Satzyperova and shifted *Wendia* and *Apiifolia* to the level of subgenus [e.g. Subgen. *Wendia* (Hoffm.) Satzyperova, Subgen. *Apiifolia* (Hoffm.) Satzyperova].

2.2.4 Conclusion

Although *Heracleum* has traditionally been divided into sections as a reflection of putative natural groupings, it is biased to Western Europe and Western Asia (de Candolle, 1830; Boissier 1872; Mandenova (1950, 1962, 1982, and 1987); Satzyperova 1984; Tamamschjan 1967; Brummit 1968). Among these only Mandenova's system can be regarded as the most comprehensive classification system for W Asian *Heracleum* (Logacheva *et al*. In press). After many years of study Mandenova (1950, 1962, 1982, and 1987) published a series of papers that put forward a classification of *Heracleum* into eight Sections: *Heracleum* L., *Villosa* Manden., *Pubescentia* Manden., *Apiifolia* Manden., *Wendia* (Hoffm.) Manden, *Vocontia* (Calest.) Thell., *Lasiopetala* Manden., and *Psedotragium* Boiss. This system was mainly based on the presence and morphology of the vittae in the fruit, with supporting characters of the petals, involucre and involucel (see Chapter 4, 6, 7 for further information).

Table 2.1 Taxonomic history of *Heracleum* L. investigated by Linnaeus, Hoffmann, Koch, De Candolle, Boissier, Mandenova, He *et al*., and Zhao *et al*.

Linnaeus (1753)	**Hoffmann (1816)**	**Koch (1824)**	**De Candolle (1830)**	**Boissier (1872)**	**Mandenova (1950, 1962, 1987)**	**He *et al*. (1998)***	**Zhao *et al.* (2004)***
Genus *Heracleum*	**Genus *Heracleum***	**Genus *Heracleum***	**Genus *Heracleum***	**Genus *Heracleum***	**Genus *Heracleum***	**Genus *Heracleum***	**Sect. Heracleum**
H. sphondylium	*H. sibiricum*	*H. sibiricum*	**Sect. *Euheracleum***	**Sect. *Euheracleum***	**Sect. (Eu) *Heracleum***	**Sect. *Heracleum***	*H. hemsleyanum*
H. sibiricum	**Genus *Sphondylium***	**Genus *Sphondylium***	*H. Flavescens*	*H. villosum*	*H. aconitifolium*	*H. hemsleyanum*	*H. vicinum*
H. panaces	*S. branca ursina*	*H. sphondylum*	*H. orsini*	*H. cyclocarpum*	*H. asperum*	*H. vicinum*	*H. moellendorffii*
H. austriacum	**Genus *Wendia***	*H. longifolium*	*H. aureum*	*H. orphanidis*	*H. carpaticum*	*H. trifolium*	*H.acuminatum*
H. alpinum	*W. chorodanum*	*H. pyrenaicum*	**Sect. *Tetrataenium***	*H. palmatum*	*H. colchicum*	*H. nyalamense*	*H. oreocharis*
		H. villosum	*H. nepalensis*	*H. lasiopetalum*	*H. cyclocarpum*	*H. moellendorffii*	*H. souliei*
		H. ligusticifolium	*H. obtusifolium*	*H. spathiphyllum*	*H. dissectum*	var. *moellendorffii*	*H. yungningense*
		Genus *Wendia*	*H. rigense*	*H. platytaenium*	*H. freynianum*	var. *paucivittatum*	*H. stenopterum*
		H. alpinum	**Sect. *Sphondylium***	*H. umbonatum*	*H. lanatum*	var. *subbipinnatum*	**Sect. *Plurivittata***
		H. austriacum	*H. longifolium*	*H. jugatum*	*H. moellendorffii*	*H. franchetii*	*H. wenchuanense*
			H. palmatilobum	*H. pubescens*	*H. ossethicum*	*H. dissectifolium*	*H. bivittatum*
			H. sphondylium	*H. persicum*	*H. platytaenium*	*H. dissectum*	**Sect. *Villosa***
			H. capsicum	*H. sphondylium*	*H. ponticum*	*H. oreocharis*	*H. obtusifolium*
			H. candicans	*H. lehmannianum*	*H. sibiricum*	*H. forrestii*	*H. candicans*
			H. lanatum	*H. crenatifolium*	*H. sphondylium*	*H. souliei*	**Sect. *Millefolia***
			H. cordatum	*H. asperum*	**Sect. *Pubesentia***	*H. scabridum*	*H. millefolium*
			H. pyrenaicum	*H. amanum*	*H. afghanicum*	*H. wolongense*	*H. longilobum*
			H. panaces	*H. sibiricum*	*H. crenatifolium*	*H. kingdonii*	**Sect. *Wendia***
			H. asperum	*H. apiifolium*	*H. lehmannianum*	*H. stenopteroides*	*H. apaense*
			Sect. *Camelia*	*H. ligusticifolium*	*H. mantegazzianum*	*H. yungningense*	
			H. carmeli	*H. pastinacifolium*	*H. rechingeri*	*H. stenopterum*	
			Sect. *Wendia*	*H. brevivittatum*	*H. sosnowskyi*	*H. henryi*	

* Mentioned in chapter 3

Table 2.1 Taxonomic history of *Heracleum* L. investigated by Linnaeus, Hoffmann, Koch, De Candolle, Boissier, Mandenova, He *et al*. and Zhao *et al.*, continued

Linnaeus (1753)	**Hoffmann (1816)**	**Koch (1824)**	**De Candolle (1830)**	**Boissier (1872)**	**Mandenova (1950, 1962, 1987)**	**He *et al*. (1998)***	**Zhao *et al* (2004)***
			H. alpinum	*H. incanum*	*H. trachyloma*	**Sect. *Plurivittata***	
			H. austriacum	*H. chorodanum*	**Sect. *Villosa***	*H. wenchuanense*	
			H. chorodanum	*H. humile*	*H. antasiaticum*	*H. bivittatum*	
			H. minimum	*H. argaeum*	*H. grandiflorum*	*H. barmanicum*	
			Sect. *Trichogonium*	*H. anisactis*	*H. leskovii*	**Sect. *Villosa***	
			H. wallichii	**Sect. *Pseudotragium***	*H. scabrum*	*H. obtusifolium*	
				H. pastinaca.	**Sect. *Wendia***	*H. candicans*	
					H. anisactis	*H. canescens*	
					H. argaeum	**Sect. *Millefolia***	
					H. chorodanum	*H. millefolium*	
					H. humile	*H. longilobum*	
					H. pastinacifolium	**Sect. *Wendia***	
					H. rawianum	*H. fargesii*	
					H. roseum	*H. subtomentellum*	
					H. schelkownikovii	*H. xiaojinense*	
					H. transcaucasicum		
					Sect. *Apiifolia*		
					H. apiifolium		
					H. ligusticifolium		
					Sect. *Lasiopetala*		
					H. lasiopetalum		
					Sect. Pseudotragium		
					H. pastinaca		
					Sect. *Vocontia*		
					H. minimum		

* Mentioned in chapter 3.

Table 2.2. Comparison of selected characters of *Heracleum*, *Semenovia*, *Tordyliopsis*, *Pastinaca*, *and Zosima.*

	Heracleum	***Semenovia***	***Tordyliopsis***	***Pastanica***	***Zosima***	***Tordylium***
Habit	Herbs, perennial rarely Biennial	Herbs, perennial, stems sometimes woody	Herbs, perennial	Herbs, biennial, perennial	Herbs, biennial or perennial, stems sometimes woody	Herbs, Annual
Stem	Fibrous collar absent	Tough fibrous collar present	Tough fibrous collar present	Fibrous collar absent	Tough fibrous collar present	Fibrous collar absent
Leaf Blade	Ternately or pinnately compound	1- to 2-pinnate	1-pinnate	1-pinnate (rarely 2-pinnate)	1- to 3-pinnate	1-to 3-pinnate
Leaflets	Broad, crenate or serrate margins	Linear to oval, dentate, lobed, or divided leaflets	Oval to obovate, coarsely dentate or trilobed	Oblong, ovate, or triangular, serrate or lobed margin	Ovate, triangular serrate or lobed margin	Ovate to cordate, Crenate to serrate margins
Bracts/ Bracteoles	Few or absent, caducous , linear or lanceolate, entire	Several, narrow, or lacking	Several, narrow, tomentose, subentire, half as long as rays, or longer	Linear-lanceolate, glabrous or hairy with ciliate margins	Linear, linear-lanceolate, ciliate margin	Filiform, linear, lanceolate, to oblanceolate-spathulate
Rays	10-40	4-15	4-10	3-22	5-40	(2-)5-20(-40)
Petals colour	White, rarely pinkish or pale-yellow,	White (rarely pale yellow)	Greenish white, purplish	Yellow, yellow-greenish, rarely pink, brown veins	White	White, pinkish or yellow
Fruit surface	Reticulate	Reticulate	Reticulate	Reticulate/Striate	Reticulate	Vesicular to tubular or strigose hairs
Fruit margin	Smooth	Smooth	Smooth	Smooth	Smooth & thickened margin	Smooth or corrugated
Vittae-dorsal	1(-2) in each furrow	1 in each furrow	1 in each furrow	1 in each furrow	1 completely filling the vallecular region	1 in each furrow
Vittae-commissural	2(-6)	2	0-4	2	2	2
Chrom. nos.	22 (44)	22 (24)	Information not available	22	(6) 12	(8, 18) 20 (22)

In this table genus *Tetrataenium* is included in genus *Heracleum*. For details, see Pu and Watson (2005), Mukherjee and Constance (1993), Menemen and Jury (2001a, 2001b), and Pimenov *et al*. (2003).

Chapter 3: *Heracleum* in the Sino-Himalayan regions

3.1 Delimitation of Sino-Himalayan floristic region

The Himalaya is an immense series of mountains that extends some 3200 km, from the Pamir Knot on the Afghan border eastwards through Jammu and Kashmir, Himachal Pradesh, Attaruncal, Nepal, Sikkim, Chumbi Valley, Bhutan, Arunchal Pradesh and the Northern tip of Burma. The Himalaya finally ends in SW China where it merges with the Hengduan mountains, a complex series of high mountains and deep valleys running north to south (www.floraofnepal.org). Some prefer a narrow definition of the Himalaya from the Indus Trench below Nanga Parbat (74˚50' E) to the Yarlungtsangpo-Bramaputra Gorge below Namche Barwa (95˚40' E) (Mani, 1978).

In the literal sense, Sino-Himalaya means China and the Himalaya, and although this is an adjacent geographic region, botanically it contains a range of floristic units with complex relationships and no clear boundaries. After Good's (1964) proposal of a Sino-Japanese floristic Region, the close affinities between the temperate floras of the Himalaya, China and Japan have long been recognized and demonstrated for many plant groups (Kanai, 1966). Takhtajan (1986) revised Good's system extensively and his classification using six Kingdoms, eight subkingdoms, 35 floristic regions, and 152 provinces is still widely in use today. For the Himalaya and adjacent area he recognized two regions, East Asiatic region, containing the Eastern Himalayan province, and Irano-Turanian region which is composed of Tibetan province and Western Himalaya province. Wu & Wu (1996) expanded on Takhtajan's system by proposing refinements of the

Eastern Asiatic Region. They promoted the Eastern Asiatic region up to Kingdom and included several subkingdoms. In this system, the Sino-Himalayan and Sino-Japanese subkingdom are separated.

To define the extent of the Sino-Himalayan Floristic region, Yoshida (2006) proposed that it should include the wetter part of the Himalaya and the mountainous part of south-western China. This covers the Hengduan Mountains and the adjacent northern highlands, and extends to Kashmir in the west and to the Qilian Mountains in Qinghai Province in the northeast (Fig. 3.1). This Yoshida's Sino-Himalayan floristic region is well characterized by the many genera with abundant species (e.g. *Saussurea*, *Pedicularis*, *Gentiana*, *Impatiens*, *Meconopsis*) and also majority of Sino-Himalayan *Heracluem* species occurs too. The present work generally follows Yoshida's (2006) floristic delimitation of the Sino-Himalaya region and includes:

1) Himalaya region: E. Pakistan, Kashmir, Himachal, Garhwal, Kumaon, Nepal, Sikkim, Bhutan, SE Tibet.
2) West China (North tip of Myanmar, NW Yunnan, Sichuan, Qinghai and East Tibet)

'The Himalaya' as used in this thesis is a broader concept that includes not only these wetter parts (Eastern Himalaya), but also the dry areas of the far west of the mountain range including W. Pakistan. The two terms 'Himalaya' and Sino-Himalayan region are therefore not synonymous.

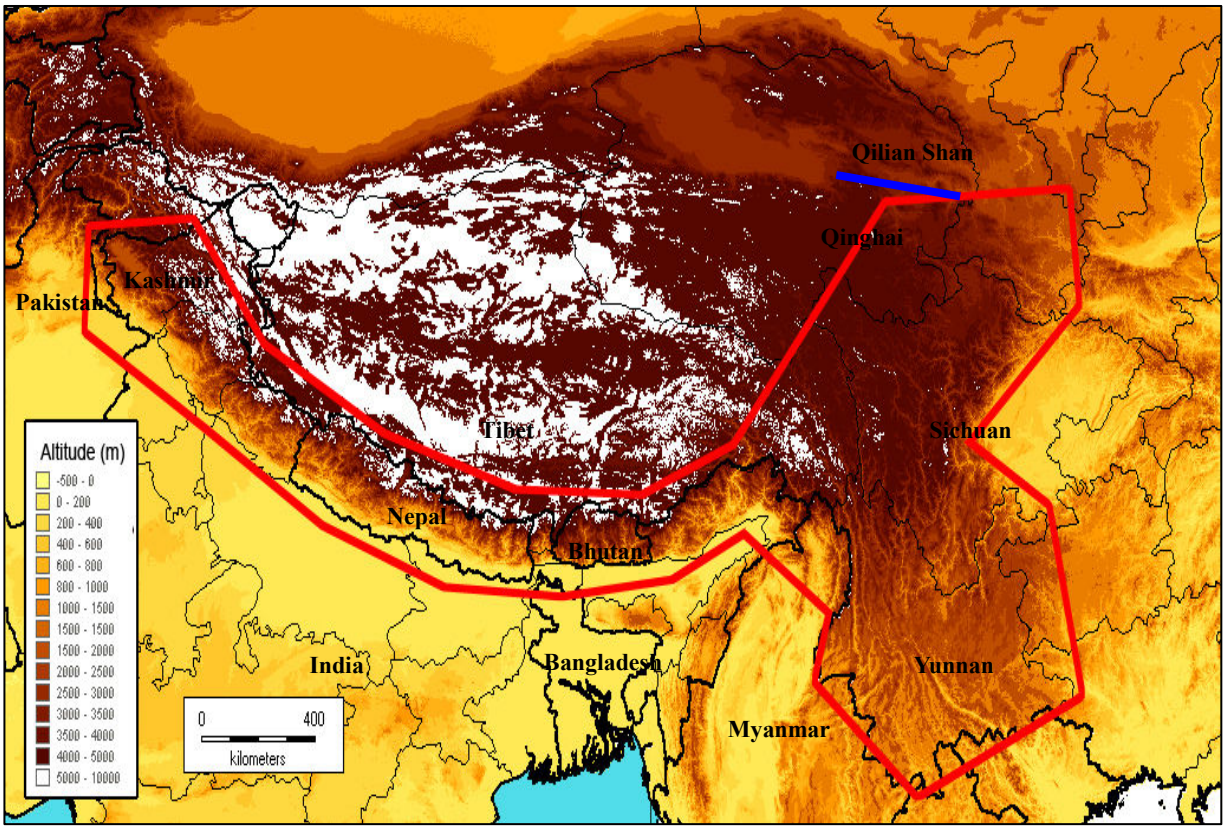


Fig. 3.1 Map of Sino-Himalayan region and major distribution area of Sino-Himalayan *Heracleum*. Around 40 species of 60(-65) species of *Heracleum* distributed this area (see details in table 3.1 & fig. 3.2.1 - 3.2.6). Red lines indicate Floristic region of Sino-Himalayan floristic region sensu Yoshida (2006).

3.2 *Heracleum* species in the Sino-Himalayan Region

Most of the Heracleum species in this study are distributed a cross several Himalayan countries, and many are endemic to the region. The following table 3.1 summarizes the list of *Heracleum* species in the Sino-Himalayan region. The vast majority of taxa, especially those in China, are still rather poorly known and understood, and many still need typification.

This study is mainly concerned with *Heracleum* taxa in this geographical area, but for a better understanding of the diversity within the genus a selection of material

from throughout the whole range of distribution (E, NE China, Korea, Japan, East Russia, North America) were also consulted.

The species concepts adopted in this study follows Nasir (1972), Mukherijee & Constance (1993) and Watson (1999) for Himalayan *Heracleum* and Pu & Watson (2005) for Chinese *Heracleum*.

Table 3.1 *Heracleum* species distributed in the Sino-Himalayan area

Taxa	Distribution	Comments
Heracleum brunonis DC. *(=Tordyliopsis)*	Bhutan, China (S Tibet)	Sometimes treated as a monotypic Himalayan genus
H. bhutanicum M.F. Watson	Bhutan	Endemic to Bhutan
H. biternatum W. Smith	Assam and Burma	Type locality: Burma at Maymyo hills, upper Burma
H. bivittatum De Boissieu	China (Guangxi, Guizhou, SW Sichuan, W Yunnan)	Vittae 2 in each lateral furrow
H. burmanicum Kurz	NE India (Khasia and Naga Hills), Myanmar	Also written "*barmanicum*" and "*birmanicum*"
H. cachemiricum C.B. Clarke	Western Himalaya (Kashmir to Pakistan)	Alava (*Flora Iranica* 161) transferred this species to *Tricholaser* Gill, as *T. cachemiricum* (C.B. Clarke) Alava
H. candicans DC.	Pakistan, India, Nepal, Bhutan, China	This species is very variable, particularly in the size and dissection of the leaves and the shape of the leaflets.
H. canescens Lindley	India, Nepal, Pakistan	Synonym *H. hirsutum* Edgew.
H. dissectifolium K.T. Fu	China (Gansu, Sichuan)	This is incompletely known species
H. fargesii de Boissieu	China (NE Sichuan)	This species reputed medicinal value, commissure without vittae.
H. forrestii H. Wolff	China (NW Yunnan)	Incompletely known species, recorded only from a few collections. Species delimitation between this and the three species (*H. hemsleyanum, H. scabridum, H. oreocharis*) from SW China is problematic.
H. franchetii M. Hiroe	China (W Hubei, Qinghai, W Sichuan, NW Yunnan)	*= H. acuminatum*
H. hemsleyanum Diels	China (Hubei, SE, W Sichuan)	Incompletely known species
H. henryi H. Wollf	China (Yunnan)	This species reputed medicinal value
H. jacquemontii C.B. Clarke	India (Western Himalaya)	Only the type collection is known
H. kingdonii H. Wollf	Myanmar, China (SE Tibet, NW Yunnan)	Easily misidentified by *H. bivittatum* or *H. burmanicum*
H. lallii Norman	Nepal	Endemic to Nepal
H. millefolium Diels	China (SE Tibet, Qinghai, W Sichuan, NW Yunnan)	This species is retained within the genus *Heracleum* in the Flora of China on account of the radiant outer flowers and clavate

		vittae
H. moellendorffii Hance	China (Broad distribution across China)	Three varieties are known (var. *subbipinnatum*, var. *moellendorfii*, var. *paucivittatum*)
H. nepalense D. Don	Eastern Himalaya, W Bengal to Nepal, Bhutan, China (NW Yunnan)	Widely distributed species
H. obtusifolium DC.	India (Eastern Himalaya), Bhutan to Nepal	This species is very variable, particularly in the size and dissection of the leaves and the shape of the leaflets.
H. oreocharis H. Wolff	China	Incompletely known species, recorded only from a few collections
H. pinnatum C.B. Clake	India, Pakistan	Plants of dry areas with rough, hispidulous pubescence
H. rapula Franchet	China	Incompletely known species, recorded only from a few collections
H. scabridum Franchet	China (SW Sichuan, NW Yunnan)	This species reputed medicinal value
H. souliei de Boissieu	China (W Sichuan)	Incompletely known species, recorded only from a few collections
H. sphondylium L.	World wide	A very variable species
H. stenopteroides H. Wolff	China (W Yunnan)	Incompletely known species, recorded only from a few collections
H. stenopterum Diels	China (W Sichuan, W Yunnan)	This species reputed medicinal value (in Yunnan)
H. sublineare C.B. Clarke	Eastern Himalaya, from Bhutan to Nepal and Tibet	Easily misidentified as *H. wallichii*
H. vicinum de Boissieu	China (NE and W Sichuan)	This species reputed medicinal value
H. wallichii DC.	Nepal, India (Sikkim), Bhutan	Similar with *H. sublineare*
H. wenchuanense F.T. PU & X.J. He	China (NC Sichuan)	This species is recorded only from the type
H. wolongense F.T. PU & X.J. He	China (NC Sichuan)	This species is recorded only from the type
H. woodii Watson	Bhutan, Nepal	Similar to *H. nepalense* but a smaller less robust plant and generally less hairy.
H. xiaojinense F.T. PU & X.J. He	China (W Sichuan)	*= Angelica apaensis*
H. yungningense Handel-Mazzetti	China (W Sichuan, NW Yunnan)	This species reputed medicinal value
H. yunnanense Franchet	China (Yunnan)	Incompletely known species, recorded only from a few collections

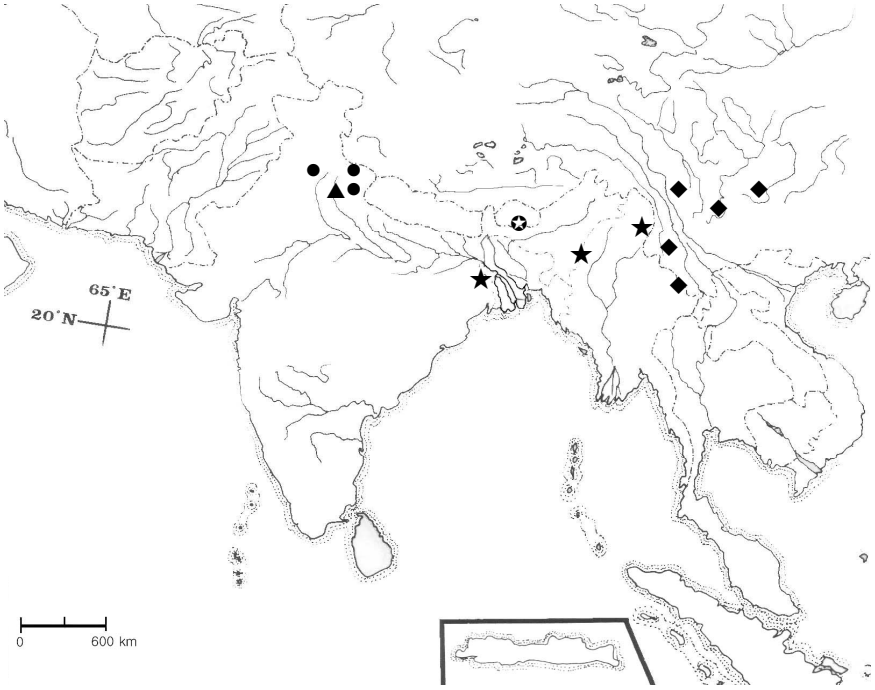


Fig. 3.2.1 Known distribution of *H. jacquemontii* C. B. Clarke (▲), *H. cachemiricum* C. B. Clarke (●), *H. bhutanicum* M.F. Watson (✪), *H. burmanicum* Kurz (★), *H. biternatum* W. Smith (◆) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

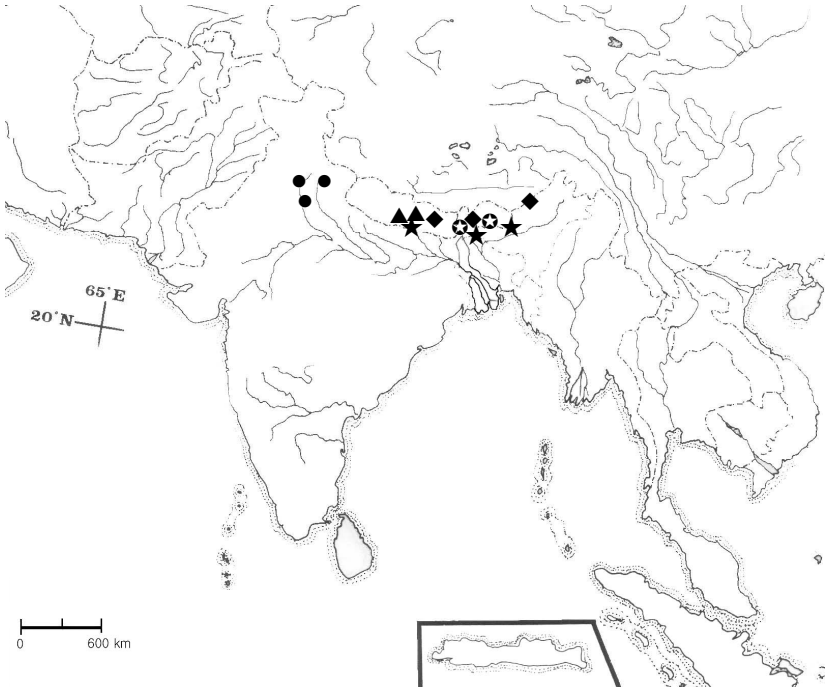


Fig. 3.2.2 Known distribution of *H. pinnatum* C. B. Clake (●), *H. woodii* Watson (✪), *H. sublineare* C. B. Clarke (◆), *H. wallichii* DC (★), *H. lallii* Norman (▲) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

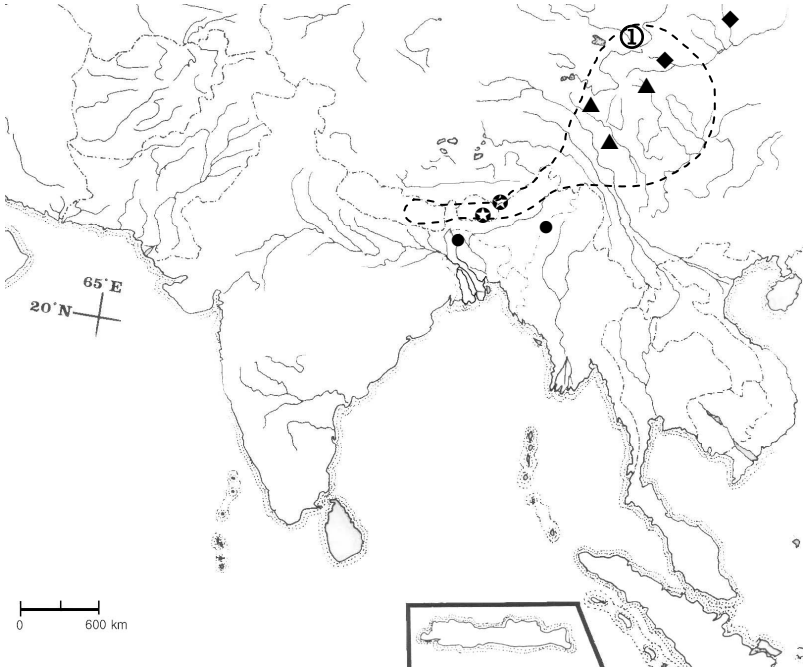


Fig. 3.2.3 Known distribution of *Tordyliopsis brunonis* DC. (✪),*Heracleum biternatum* W. Smith (●), *H. dissectifolium* K. T. Fu (◆), *H. franchetii* M. Hiroe (▲), *H. obtusifolium* DC. (①) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

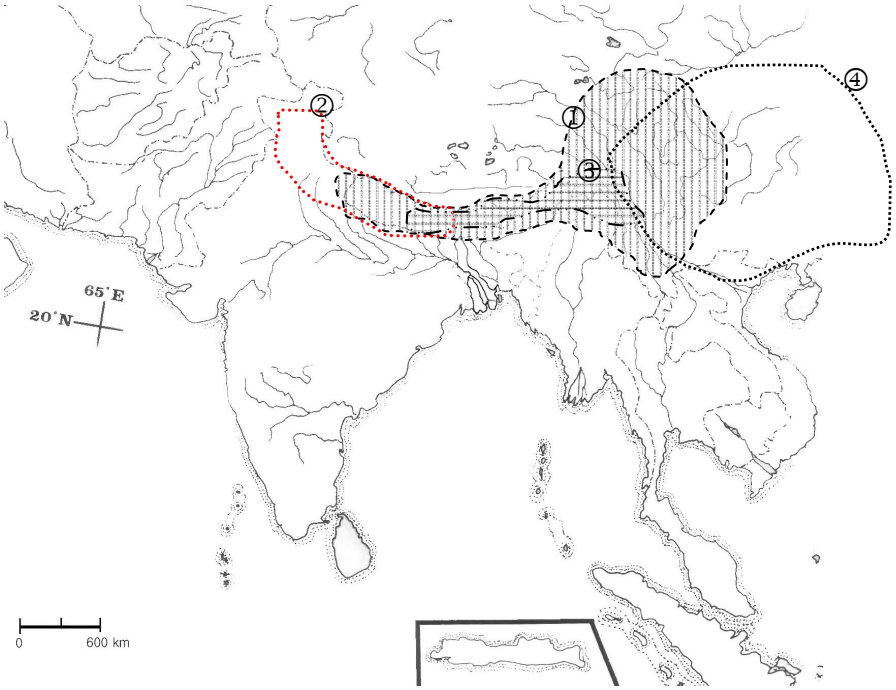


Fig. 3.2.4 Known distribution of *H. candicans* DC. (①), *H. canescens* Lindley (②), *H. nepalense* D. Don (③), *H. moellendorffii* Hance (④) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

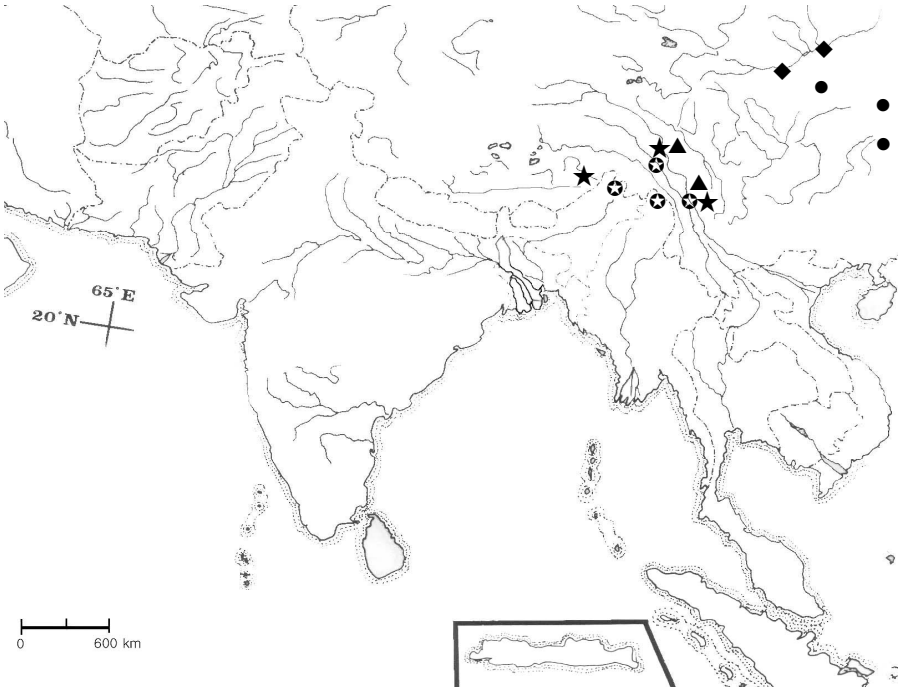


Fig. 3.2.5 Known distribution of *H. fargesii* de Boissieu (◆), *H. forrestii* H. Wolff(★), *H. hemsleyanum* Diels (●), *H. henryi* H. Wollf (▲), *H. kingdonii* H. Wollf (✪) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

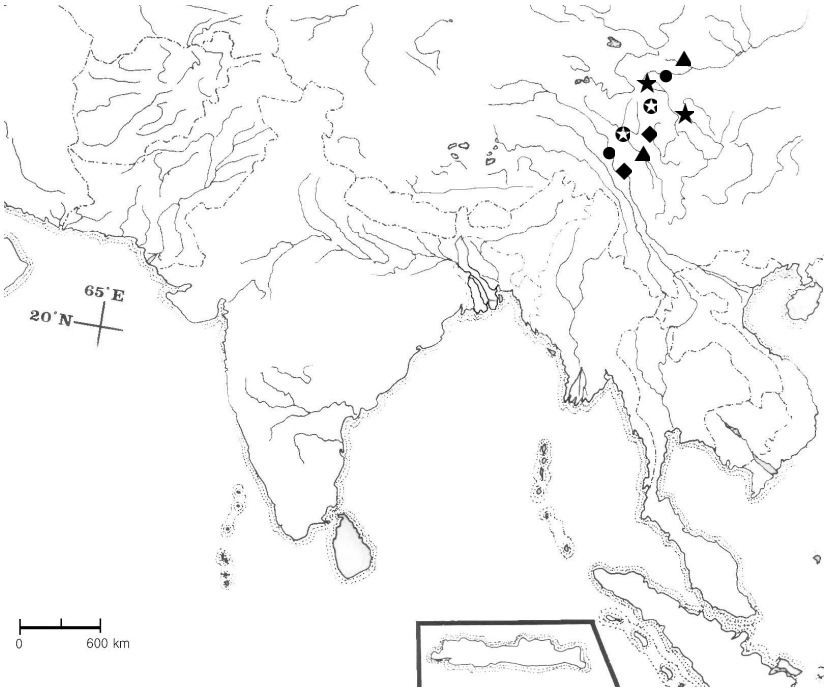


Fig. 3.2.6 Known distribution of *H. oreocharis* H. Wolff (●), *H. scabridum* Franchet (▲), *H. souliei* de Boissieu (★), *H. stenopterum* Diels (✪), *H. yunnanense* Franchet (◆) in the Sino-Himalaya region (also in Russia, Mongolia, the Middle East and W C & S Europe).

3.3. Historical survey of Sino-Himalayan *Heracleum*

Compared to the western parts of Eurasian our understanding of Sino-Himalayan *Heracleum* has suffered through lack of detailed systematic study and paucity of good herbarium collections. De Candolle (1830) was the first to include Himalayan species in a taxonomic revision of *Heracleum*. In *Prodromus Systematis Naturalis* , he divided the 26 species of the genus known at that time into six sections based on the number of channels on the commissural of the fruit. He included four species from the Himalaya, placing them in two new sections: Sect. *Tetrataenium*, including *H. nepalensis*, *H. obtusifolium*, *H. regense*; and Sect. *Trichogonium*, including just *H. wallichii*. Later Section *Tetrataenium*, which by then was expanded to include more species mainly from the Central Himalayan and South India, was elevated to generic rank by Mandenova (1959, 1982 and 1987).

The first complete treatment of *Heracleum* in the Central Himalaya and India was that carefully written by Charles Baron Clarke (1879) for J. D. Hooker's *Flora of British India*. His account of *Heracleum* recognized 21 species which he characterised only on plant gross morphology, fruit colour and pubescence.

Since Clarke's classic work, members of the genus have been treated in local Floras and a number of new taxa have been reported. Important contributors for the Himalaya region were Gamble (1919) and H. Wolff (1910-1930) *Heracleum* has been treated in the *Flora of Pakistan* (Nasir, 1972), and *An Enumeration of the Flowering Plants of Nepal* by Cannon (1978). Cannon

provided a critical and most helpful list of *Heracleum* of Nepal. The most recent assessment of *Heracleum* covering the whole of India and Central Himalaya region is the *Umbelliferae of India* by Mukherjee & Constance (1993). In this account 20 *Heracleum* species were recognized and *Tetrataenium* was included within *Heracleum* because the authors decided that the characteristics of *Tetrataenium* (Mandenova, 1987) are not clearly defined and much more population level investigations are needed. Watson's treatment of *Heracleum* in the *Flora of Bhutan* (Watson, 1999) covered seven species of *Heracleum,* including two new species (*H. butanicum* and *H. woodii*) in Bhutan and part of Sikkim.

Many Chinese plants were introduced into Europe by plant hunters collecting at the turn of the 20th century (Lauener, 1996). The Chinese specimens of *Heracleum* gathered by these famous collectors form the basis of many of the more recent works on the genus. Important contributors were Augustine Henry, Soulie, and George Forrest, Frank Kingdon-Ward, E.H. Wilson and the French Missionaries Père David, Farges and Delevay (Lauener, 1996). The classification of Chinese *Heracleum* has been attempted several times and they have been included in several European works (e.g. Franchet, 1894; de Boissieu, 1903, and Wolff, 1933-34). Since then, regional Floras have been published on this subject but without many changes to the classification. A most encouraging recent development was the appearance of the *Flora Reipublicae Popularis Sinicae* (Shan & Shen, 1992) and slightly modified and developed treatment for the *New English edition of Flora of China* (Pu & Watson, 2005). In here *Heracleum* classification was revised for parts of the range of the genus and well illustrated,

However, this account followed mostly traditional classification, and did not propose many radical changes. In parallel with this, He *et al*. (1998) revised the system of Chinese *Heracleum* for the first time based on a combination of fruit morphology, anatomy, pollen morphology, cytology and anatomical characters of petiole. The 29 species of *Heracleum* in China were divided into five sections: Sect. *Wendia*, Sect. *Heracleum*, Sect. *Villosa*, Sect. *Millefolia* and a new Sect. *Plurivittata*). However, the samples used in this study were too small and identification of some species was ambiguous, thus, the new system could not be adopted in the most recent English version of *Flora of China* account.

The taxonomy of *Heracleum* in the Sino-Himalayan region remains problematic with difficulties in delimiting taxa at all ranks from genera to species and below. This is not only the species only known from the type or one or two incompletely known collections also the range of variation within and between individuals and populations is poorly understood as taxonomists have largely had to rely on herbarium specimens for their study. Many of the species are large plants and when they are collected the specimens are often incomplete or lack crucial characters. The paucity of good herbarium collections and tendency for traditionally significant characters to degenerate in the herbarium adds to the problems as brittle leaves and bracteoles can be easily damaged in transit and storage. As basal leaves are often very large, they are seldom preserved, so all significant characters relating to them are lost for many collections.

The Sino-Himalayan region is very important to the understanding of *Heracleum* and its related genera. The East Himalaya/SW China region is one of two centres

of diversity of this widespread genus. Also, problems of morphological complexity and blurred generic boundaries in this region highlight its importance. As outlined in Chapter 2, the generic characters traditionally used to recognise *Heracleum* in the western part of its range do not work well in the Sino-Himalayan region. *Heracleum* species (e.g. *H. burmanicum*, *H. biternatum*) In SE Asia area (Tropical south India, Upper Burma, Thailand) have fruits that are quite unlike those of the Caucasian species, but the Sino-Himalayan species appears to provide a grade between these two areas (Hedge & Lamond, 1992). Consequently, the Sino-Himalayan species are crucial in a world understanding for *Heracleum* as it is here that the problems of generic delimitation are most acute.

The following chapter provides an overview of the morphology of *Heracleum* in the Sino-Himalayan floristic region and attempts to identify the most important characters for study of the genus in this area.

Chapter 4: Taxonomic Characters

4.1 General morphology

Literature relevant to the *Heracleum* and related genera worldwide was surveyed (Brummitt, 1968; Mandenova, 1987) with particular attention to those pertaining to Himalayan species (Mukherjee & Constance, 1993; Nasir, 1972; Watson, 1999) and Chinese species (Pu & Watson, 2005; Shan & Sheh, 1992) including Thailand (Hedge & Lamond, 1992). This survey tried to determine which characters had been previously useful in the identification and delimitation of species worldwide and particularly in the Sino-Himalayan area. Herbarium specimens collected from the Sino-Himalayan floristic region were also studied to assess these application and usefulness of these characters and search for novel potentially useful taxonomic characters. Loans of herbarium materials from BR, CAS, E, G, K, MO, NY, KUN, P, PE, UPS, US, and WU were arranged (abbreviations follow Holmgren & Barnett, 1990). As well as consulting all the material at E, study visits to the herbaria at BM, NAS, K, KUN and P were made and selected material from these was also consulted on loan. Specimens belonging to all the taxa and countries listed in chapter 3 (including chapter 3, table 3.1) were examined. Where possible they were examined with a 20 – 25x binocular dissecting microscope to determine character states and confirm findings of the surveyed literature. Selected characters are summarized in table 4.1. Eight species of *Heracleum* from RBGE SeedBank were sown and cultivated in the greenhouses of RBGE. After successful germination (two species: *H. candicans*, *H. pinnatum*) the seedling development process were observed. Field

observations of *Heracleum* species in the wild were conducted in Gaoligongshan (Old Sibali & Chukai area, Yunnan, China) and Edinburgh. In particular, variation in basal leaf dissection, leaflet size, bracts and bracteole size and numbers, flower colours in different habitat were observed. The plants were identified based on published Floristic accounts and in comparison with verified herbarium specimens at E and on loan from other Herbaria. General terminologies for this chapter and following chapter follow Kljuykov *et al*. (2004)

Published standard floras are as follows:

- *Flora Europaea* (Brummit, 1968)
- *Flora Iranica* (Mandenova, 1987)
- *New English Edition of Flora of China* (Pu & Watson, 2005)
- *Flora of China* (Shan & Sheh, 1992)
- *Flora of Bhutan* (Watson, 1999)
- *Umbelliferae of India* (Mukherjiee & Constance, 1993)
- *Flora of Thailand* (Hedge & Lamond, 1992)

Table 4.1 Selected characters of general morphological characters of Sino-Himalayan *Heracleum*.

Taxa	Height (cm)	Cauline leaf sheath	Basal leaves division	Leaflet shape	Calyx teeth shape	Fruit wing vs body[1]	Dorsal & lateral ribs distribution	Vittae form	Vittae size ratio[2]
H. bhutanicum M.F. Watson	70- 100	Narrowly sheathing	1-2 pinnate	Ovate-lanceolate/ narrowly ovate	Obsolete	Equal or slightly narrower	Close	Linear	3/4
H. bivittatum de Boissieu	80 - 100	Narrowly sheathing	2-pinnate	Ovate-oblong	Prominent, triangular	Wider than body	Close	Linear	3/4-4/4
H. cachemiricum C.B. Clarke	60 (-100)	Inconspicuous	2-pinnat, ternate-pinnate	Lineary laceolate to oblong	Obsolete	Narrower than body	Close	Linear	3/4
H. candicans DC.	80 -100 (-200)	Conspicuously Sheathing	pinnate/ ternate-pinnate	Oblong to ovate	Prominent, linear	Narrower than body	Close	Linear	3/4
H. canescens Lindley	80 - 100	Inconspicuous	pinnate	Oblong to ovate	Prominent, linear	Equal or slightly narrower	Close	Linear	2/3
H. fargesii de Boissieu	60 - 80	Moderately sheathing	ternate	Ovate	Prominent, lanceolate	Narrower than body	Wide	Clavate	1/2
H. forrestii H. Wolff.	80 - 100	Narrowly sheathing	pinnate	Ovate or broad - ovate	Obsolete, minute	Narrower than body	Wide	Clavate	3/4
H. franchetii M. Hiroe	80 - 100	Narrowly sheathing	(1-)2-pinnate	Ovate-oblong or lanceolate	Prominent, triangular	Narrower than body	Wide	Clavate	1/2

[1]: comparison of width between fruit wing and body; [2]: ratio between vittae and mericarp size.

Table 4.1 Selected characters of general morphological characters of Sino-Himalayan *Heracleum*, continued (1)

Taxa	Height (cm)	Cauline leaf sheath	Basal leaves division	Leaflets shape	Calyx teeth shape	Fruit wing vs body[1]	Dorsal & lateral ribs distribution	Vittae form	Vittae size ratio[2]
H. hemsleyanum Diels	100 - 150	Narrowly sheathing	Pinnate	Ovate or lanceolate	Obsolete	Narrower than body	Wide	Clavate	1/2
H. henryi H. Wollf	60 - 80	Narrowly sheathing	ternate	Ovate-oblong or ovate-lanceolate	Prominent, lanceolate	Wider than body	Close	Linear	3/4
H. kingdonii H. Wollf	50 - 90	Moderately sheathing	1-2-pinnate	Ovate, ovate-oblong	Prominent, triangular	Wider than body	Close	Linear	1/2
H. lallii Norman	60 - 90	Moderately sheathing	pinnate	Ovate	Obsolete	Narrower than body	Close	Sub clavate	2/3
H. millefolium Diels	10 - 30 (- 50)	Narrowly sheathing	3-4-pinnate	Linear	Prominent, triangular	Narrower than body	Wide	Linear	3/4
H. moellendorffii Hance	80 -150 (-200)	Moderately sheathing	Ternate or ternate-pinnate	Broadly ovate	Obsolete	Narrower than body	Wide	Sub clavate	1/2
H. nepalense D. Don	100 - 150 (-250)	Inconspicuous	pinnate/ ternate-pinnate	Ovate	Prominet, linear	Wider than body	Close	Sub clavate	1/2-2/3
H. obtusifolium DC.	80 - 100	Inconspicuous	Pinnatifid to simply pinnate	Ovate-orbicular	Prominet, linear	Wider than body	Close	Linear	3/4

[1]: comparison of width between fruit wing and body; [2]: ratio between vittae and mericarp size.

Table 4.1 Selected characters of general morphological characters of Sino-Himalayan *Heracleum*, continued (2)

Taxa	Height (cm)	Cauline leaf sheath	Basal leaves division	Leaflets shape	Calyx teeth shape	Fruit wing vs body[1]	Dorsal & lateral ribs distribution	Vittae form	Vittae size ratio[2]
H. oreocharis H. Wolff	60 - 80	-	Pinnate	Ovate	Obsolete	Narrower than body	Wide	Clavate	2/3
H. pinnatum C.B. Clake	80 - 100	Moderately sheathing	pinnate	Oblong-oval to orbicular	Obsolete	Narrower than body	Close	Sub clavate	2/3 - 3/4
H. rigens DC.	80 - 120	Conspicuously Sheathing	pinnate	Oval to obovate	Prominent, ovate	Narrower than body	Close	Linear	3/4
H. scabridum Franchet	80 - 100	Narrowly sheathing	pinnate	Ovate or broad-Ovate	Obsolete	Narrower than body	Wide	Clavate	2/3
H. souliei de Boissieu	80 - 100	Narrowly sheathing	2-pinnate	Ovate-lanceolate	Minute, triangular	Narrower than body	Wide	Clavate	1/2
H. stenopterum Diels	80 - 120	broad-ovately sheathing	2-3-pinnate	Lanceolate	Minute, lanceolate	Narrower than body	Wide	Clavate	1/2
H. sublineare C. B. Clarke	20 - 45	Slightly sheathing	ternate-pinnate	Lineary laceolate	Prominent, linear	Narrower than body	Close	Linear	3/4
H. vicinum de Boissieu	80 - 100	Narrowly sheathing	ternate	Broadly ovate	Prominent triangular	Narrower than body	Wide	Clavate	1/2

[1]: comparison of width between fruit wing and body; [2]: ratio between vittae and mericarp size.

Table 4.1 Selected characters of general morphological characters of Sino-Himalayan *Heracleum*, continued (3)

Taxa	Height	Cauline leaf sheath	Basal leaves division	Leaflets shape	Calyx teeth shape	Fruit wing vs body[1]	Dorsal & lateral ribs distribution	Vittae form	Vittae size ratio[2]
H. wallichii DC.	60 - 80	Inconspicuous	1(-2) pinnate	Ovate-lanceolate/ oblong-elliptic	Prominet, linear	Wider than body	Close	Sub clavate	1/2
H. woodii M.F.Watson	80 - 100	Slightly sheathing	pinnate	Ovate	Prominet, triangular	Equal or slightly narrower than body	Close	Linear	2/3
H. yungningense Handel-Mazzetti	80 - 100	Moderately sheathing	2-3-pinnate	Ovate-lanceolate	Minute, triangular	Narrower than body	Wide	clavate	1/2

[1]: comparison of width between fruit wing and body; [2]: ratio between vittae and mericarp size.

4.1.1 Habit and Rootstock

All Sino-Himalayan *Heracleum* are perennial herbs, rarely biennial, mostly growing in moist conditions or, less commonly, in dry areas (e.g. *H. pinnatum*). The plant height at maturity ranges from 10 cm (e.g. *H. millefolium*) to 250 cm (e.g. *H. nepalense*). The general form of the **root** is a taproot, which is thickened, fusiform or cylindrical, rarely fascicled. Fibres from previous year's leaf bases can occur at the top of the rootstock at the crown of the plant (e.g. *H. millefolium*). Unfortunately, it is common that herbarium specimens lack roots as they are either not collected or have become detached from the specimens. Therefore, it is hard to use root characters.

4.1.2 Stem

The stem is erect, terete and often ribbed or striate, and usually branched, at least above. The **indumentum** is rather variable, from glabrous to hispidulous, hirsute, villose or tomentose pubescence. Characteristics of the **stem**, such as size and thickness (stout or slender: there are no accurate definition provided in any reference for these characters but may be less than 0.6 cm in personal measurements) are sometimes used when defining the species in combination with other morphological characters. For example, the slender stems of *H. wallichii* and *H. canescens* are particularly noteworthy (Mukherjee & Constance, 1993).

4.1.3 Leaf

In most species both basal leaves and cauline leaves are present. Basal leaves are often used in delimiting species (see below), but are not always well represented on specimens. Cauline leaves are usually reduced upwards (the uppermost often greatly

reduced) and so should be used with caution: they may be simple or sometimes similar to the compound basal leaves (e.g. *H. lallii*, *H. millefolium*). **Petiole**: the basal leaves are petiolate in nearly all species studied. Generally basal leaves have a longer petiole than cauline ones. **Petiole sheaths** are often conspicuously inflated, but may be moderately inflated or even inconspicuous (e.g. *H. cachemiricum*, *H. canescens*, *H. nepalense*, *H. obtusifolium*). The uppermost leaves may be reduced to just a sheath with a small blade.

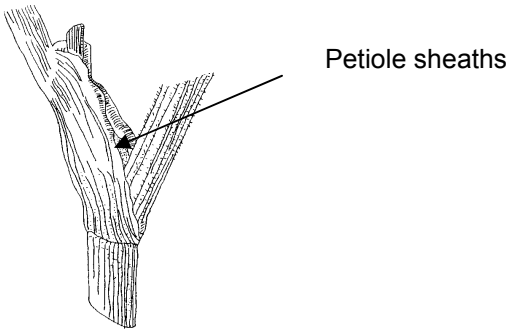


Fig. 4.1 Petiole sheaths diagram of *Heracleum*

4.1.4 Basal Leaf dissection

The basal leaves are ternately or pinnately compound or merely lobed to pinnatifid. The degree of basal leaf dissection has been used to characterize several species. It ranges from ternate leaves to 1-4 pinnate leaves (Pu & Watson, 2005). The pattern of dissection is generally constant within a species, but the degree of dissection is variable with large specimens generally having more dissected leaves. According to field research (personal observation, population study of *H. forrestii* in Gaoligongshan, Yunnan, China), considerable variation in leaf size and pinnation which corresponds to different habitats (e.g. high altitude & open steep slope: reduced size, less dissected; damp, flat and/or shaded area: enlarged size, more dissected) was found.

In shaded environments, large-leaf species may be well adapted for the conservation of water because of a thick leaf boundary air layer which reduces transpiration in comparison to small leaves (Gates & Papian, 1971; Smith & Geller, 1980; Geller & Smith, 1982).

Givnish (1978) insisted that compound leaves appear to be adaptive in at least two sorts of environmental contexts: in warm, seasonally arid situations that favour the deciduous habit, and in light gap and early successional vegetation where rapid upward growth and competition for light favour the cheap throwaway branch. Thus, compound or deeply dissected leaves can cover a large area rather inexpensively, particularly when conditions call for effectively small leaves.

The structure of leaves has important implications for the performance of plants in specific habitats. For this reason, it has been argued that it could be used to characterize species, define functional groups and predict the response of species to various environmental factors (Westoby, 1998; Weiher *et al.*, 1999). In *Heracleum* basal leaves have been considered taxonomically important, as in the *Flora of China* (Pu & Watson, 2005). However, a high degree of heteromorphism is reported and observed in Apiaceae and many other taxa. Even within a small population of *H. sphondylium* growing along a short stretch of disused railway there is considerable variation in leaf pinnation (personal observation, Edinburgh). Younger leaves, or leaves from smaller specimens are typically less dissected or ternate while in large specimens or on older leaves the pattern is more dissected. Furthermore, the leaf size is variable in most species, probably due to environmental factors with lamina of shaded leaves often broader than those in full sun. Ontogenic heteromorphism in leaf form has also been reported e.g. from *Pastinaca* (Menemen & Jury, 2001a) with successive years'

leaves changing from simple to ternate to pinnate. Therefore, it should be careful to use basal leaf shape features as diagnostic characters or when establishing relationships.

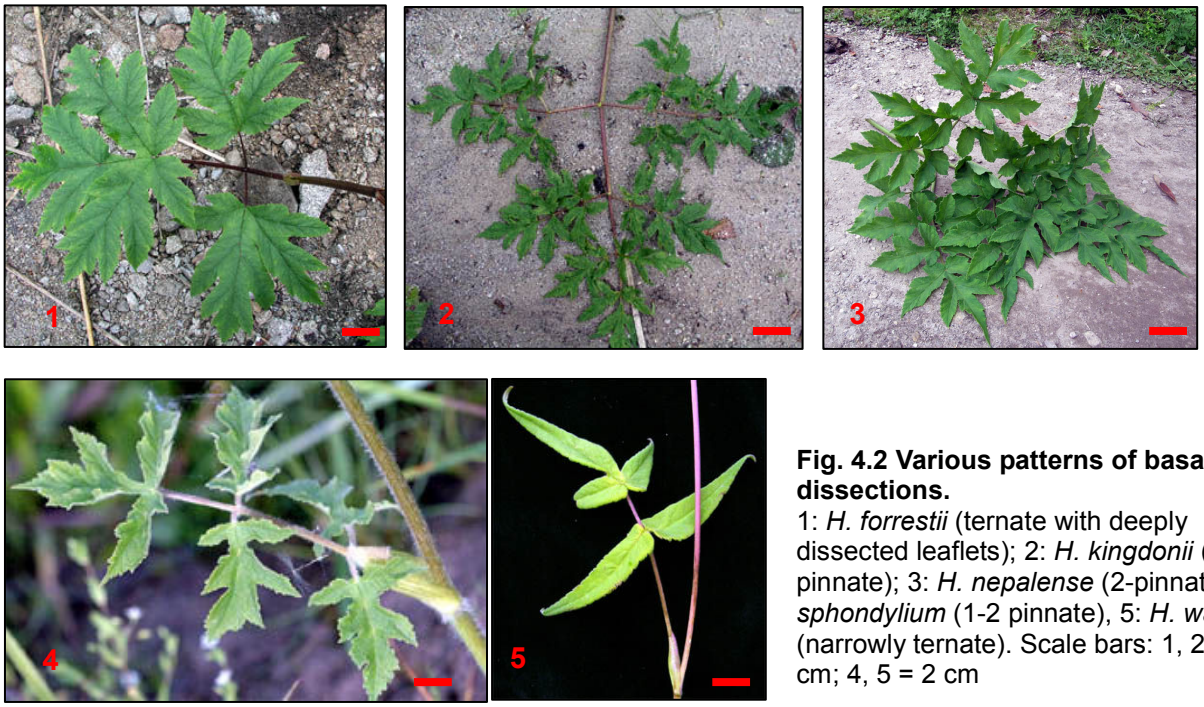


Fig. 4.2 Various patterns of basal leaf dissections.
1: *H. forrestii* (ternate with deeply dissected leaflets); 2: *H. kingdonii* (2-3 pinnate); 3: *H. nepalense* (2-pinnate), 4: *H. sphondylium* (1-2 pinnate), 5: *H. wallichii* (narrowly ternate). Scale bars: 1, 2, 3 = 5 cm; 4, 5 = 2 cm

4.1.5 Leaflets

In general the leaflets are petiolulate, but sometimes sessile. They are linear-lanceolate to oblong, oblong to broadly ovate. The margins range from broadly dentate, crenate, serrate, variously cleft or lobed, hairy (at least abaxial side) or glabrous. In the *Flora of Bhutan*, *H. candicans* and *H. obtusifolium* are distinguished on dense tomentose hairs on the undersides of the leaves (Watson, 1999).

4.1.6 Umbels

Heracleum species in this study have compound terminal and lateral umbels, the terminal umbels are usually with bisexual flowers (hermaphrodite), the lateral (secondary) umbels with mostly staminate (andromonoecy). **Bract** number from absent to few, linear, lanceolate, and are often caducous. **Rays** are few to numerous, unequal, spreading-ascending. The number of rays has been considered significant for distinguishing species (Pu & Watson, 2005). However, even if this character is obvious in several species (e.g. *H. nepalense*), the ray numbers should be used with great care because the herbarium specimens can easily have functionally male flowers. Terminal and lateral umbels can have different number of rays. **Bracteoles** are linear or lanceolate, entire, several, persistent, or less commonly caducous or lacking. Sometimes, presence of bracteole and relative size (shorter, equal, or longer than pedicels) are used for delimiting species (Boissier, 1872; Mukherjee & Constance, 1993; Pu & Watson, 2005). In *H. oreocharis*, the bracteoles equal or are slightly longer than the pedicels characterises this species (Pu & Watson, 2005). However, according to field research (personal observation, population level of comparative *Heracleum* species in Gaoligongshan, Yunnan, China), the bracteole number and size is seen to be very variable within a population and it seems to be dependent on environmental conditions.

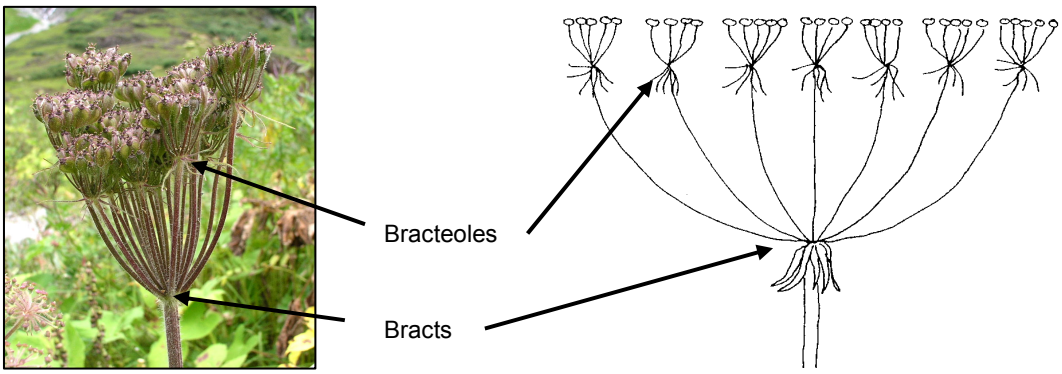
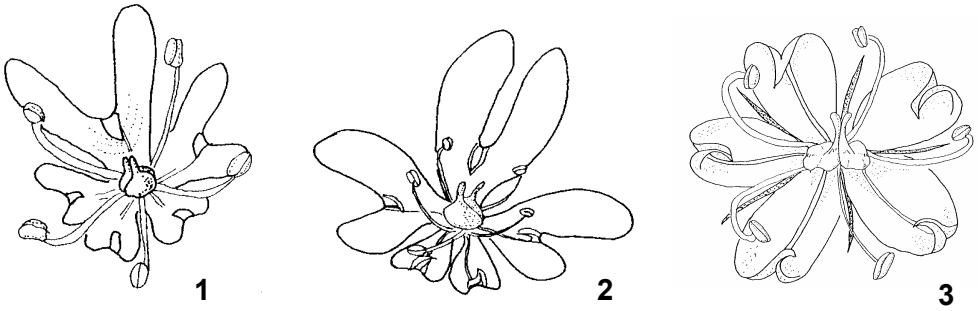


Fig. 4.3 Compound umbel structure of *Heracleum*

4.1.7 Umbellules

Umbellules vary from few- to many-flowered. The flowers are pedicellate. **Calyx teeth** are either prominent linear to lanceolate or triangular or obsolete. Mandenova (1978) included several *Heracleum* species within *Tetrataenium* on the basis of well-developed calyx teeth, better developed and narrowly distributed dorsal and lateral ribs of the fruit, and elongate, truly claviform and septate dorsal vittae. Among those characters, she considered the presence and shape of the calyx teeth to be one of the useful key characters for *Tetrataenium*. The species included within *Tetrataenium* (*sensu* Mandenova) have a general tendency to be distinct from other *Heracleum* species using the above characters, but after a careful study of expanded number of herbarium specimens in the present study it was seen that the calyx teeth characters are less dependable than was once thought, and calyx teeth development depends upon fruit position and fertility. Fruits from outer flowers usually have prominent persistent calyx teeth but in the inner sterile fruit they are sometimes evident or maybe obsolete. Even in *Heracleum* species, excluding *Tetrataenium*, the outer fruit of several species have prominent persistent calyx teeth (*H. fargesii*, *H. vicinum*, *H. henryi*). So this character must be used with care and in combined with other morphological characters. The **petal colour** is usually white or rarely pinkish or pale yellow. Although it has been considered significant in the past, and even relatively recently (Watson, 1999), petal colour is a difficult character to use as older herbarium specimens will typically have brownish cream petals. **The shape of the petal** is lanceolate to obovate or obcordate with narrow inflexed apex and cuneate base. **The outer petal** is radiant, enlarged, broadly obovate, apex deeply 2-lobed. The stylopodium shape is conical to low conical, rarely depressed. **The styles** are short, erect or reflexed. **The ovary** ornamentation is

usually pubescent or rarely glabrous.



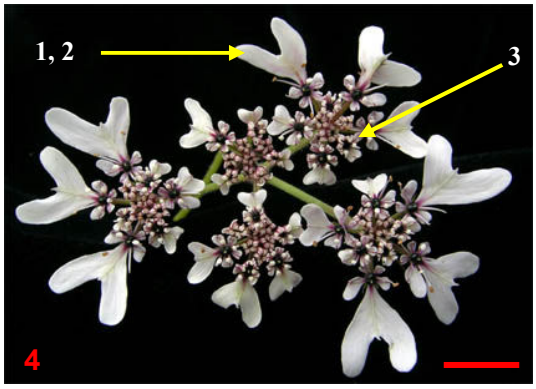


Fig. 4.4 Umbellules structure of *Heracleum.*
Diagram **1**, **2** indicate radiant flowers and **3** indicates non-radiant flower. **4** shows photographs of umbellules of *Heracleum sublineare* (1,2: radiant flower; 3: non-randiant flower). Scale bar = 1 cm

4.1.8 Fruit

The outline **shape** of the fruit is broadly ovate, elliptic, orbicular or broadly obovate, and they are strongly compressed dorsally in transverse section. The fruit apex is usually rounded or emarginate, and the base is rounded or narrowed. **Fruit ornamentation** is pubescent to glabrous. Dorsal (median) and lateral (intermediate) **ribs** are filiform, sometimes keeled, low-keeled, or obsolete. Marginal ribs are winged: the ratio of fruit wing size to that of the fruit body and the distribution of dorsal and lateral ribs (widely spaced/ narrowly spaced) are good characteristics of several species (e.g. *H. wallichii*, *H. nepalense*, *H. burmanicum*, *H kingdonii*; Mukherjee & Constance,1993; Pu & Watson, 2005).

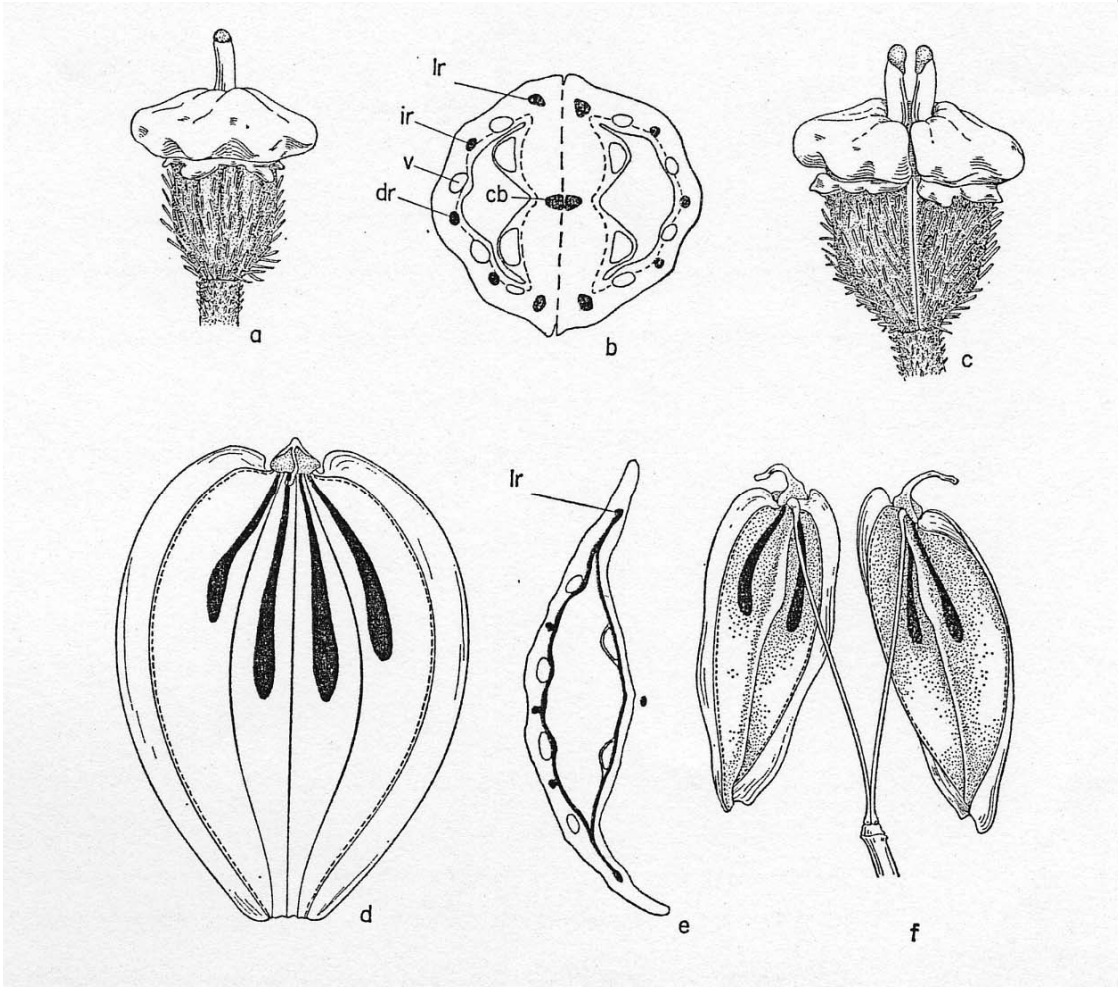


Fig. 4.5 Floral and fruit morphology and anatomy of *Heracleum lanatum* Michx.

(a) Dorsal view of flower shortly after anthesis; (b) median transition (diagrammatic) of developing mericarp shortly after anthesis; (c) lateral view of flower shortly after anthesis; (d) dorsal view of mature fruit; (e) median transaction (diagrammatic) of mature mericarp; (f) lateral view of mature fruit. [Details: *cb*, carpophore vascular bundles; *dr*, dorsal rib bundle; *ir*, intermediate rib bundle; *lr*, lateral rib bundle; *v*, vitta (secretory canal).] Figures from Theobald (1971).

4.1.9 Vittae

From a dorsal view of the vittae (oil canals) are usually prominent but occasionally obsolete. They are often clavate and much shorter than mericarp, or subclavate to linear usually reaching to base, rarely shorter than mericarp . **The number of vittae** is 1 (rarely 2 or 3) in each furrow, usually 2 (rarely to 6) or absent on the commissure. As mentioned in chapter 2, distinctive clavate vittae shorter than the length of the mericarp, are characteristic of many *Heracleum* species in the western parts of Eurasia (Brummitt, 1968). Based on our increased number of herbarium specimens, this study confirmed that this character degenerates in many Sino-Himalayan *Heracleum* species, an idea that was previously proposed by Hedge & Lamond (1992). Vittae shape, size and number are so much used in traditional classification (De Candolle 1830; Mandenova 1950, 1962, and 1986) and, in fact, there are some general tendencies within species. However these features are mostly constricted to European and W Asian *Heracleum* taxa and can not be fully applicable to Sino-Himalayan taxa (See table 4.1).

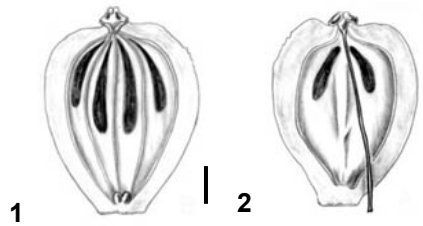


Fig. 4.6 Dorsal view of typical *Heracleum* mericarps.
1: Dorsal (front) side, **2**: Commisure (back) side.
Scale bar =0.5 cm

4.1.10 Seedings

A cultivation experiment of Himalayan species was conducted in the first time. Eight species of *Heracleum* which were collected in the wild previous expedition deposited in the RBGE SeedBank were tested and among them only two species successfully germinated. After germination *Heracleum candicans* (RBGE Acc. No.: 19943958) and *H. pinnatum* (RBGE Acc. No.: 19942979) have been observed (Fig. 4.7). The species studied have the cotyledons are linear or narrowly elliptic in shape and somewhat rounded at the apex. The blade of cotyledons is usually glabrous. The first true leaves are opposite with an ovate or obovate blade. Considering the seedling characters in *Heracleum* was done by Satzyperova (1984). She conducted seedling experiment of 33 species which are mostly from European and West Asian *Heracleum* species. All material has narrowly oblong to linear cotyledons but has variation in length. Despite only two seedling experiment was succeeded, there was no significant difference in the rate of germination with Satzyperova's experiment.

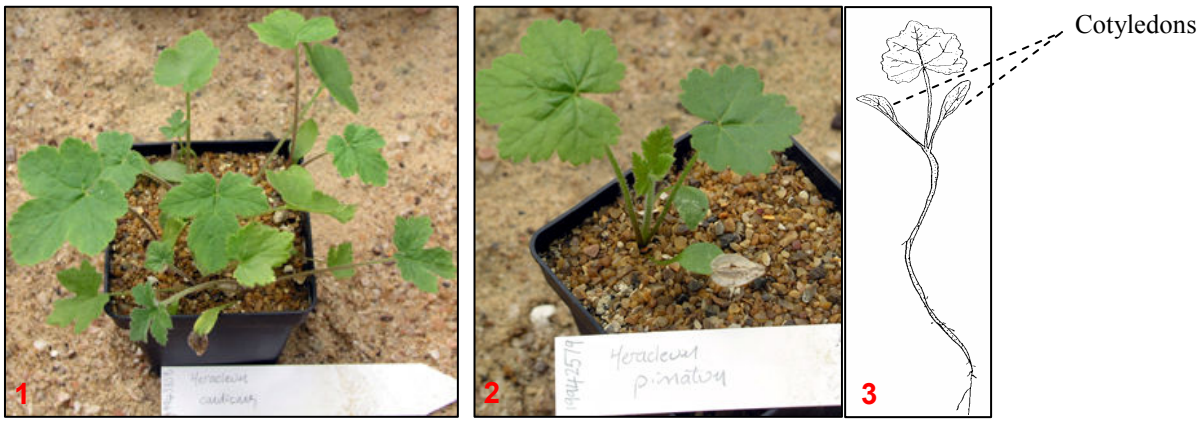


Fig. 4.7 Seedings of *Heracleum* species.

1: *Heracleum pinnatum* (Acc. No.: 19942979); **2**: *Heracleum candicans* (Acc. No.: 19943958); **3**: Diagrams of seedlings.

4.2 Summary

Following a literature and specimen survey of the above characters, I have found the following to be potentially useful for separating taxa:

- Stem size (stout/slender)
- Cauline leaf sheath extent
- Fruit wing development (fruit wing vs body ratio), and
- Dorsal and lateral rib distribution.

However, most of the species do not show singular distinguishing apomorphies, but rather it is a combination of several particular characters and character-states that have to be used to discriminate the species from each other. For the establishment of relationships within *Heracleum*, reliable and distinct characters that are shared by different species are needed. Unfortunately, there are very few of these morphological characters in *Heracleum*, therefore, macro-morphology does not provide many clues to define ‘natural’ groups. Also several characters, particularly basal leaf shape show considerable variation within a single species, and should be used with care when delimiting taxa.

In an attempt to identify further useful characters for taxon delimitation in the Sino-Himalayan *Heracelum*, I investigated macro- and micro-morphology further. Details of these studies are presented in the following chapters, and their correlation with independently determined molecular DNA phylogeny is presented in chapter 8.

Chapter 5: Leaf Micro-morphology (pilot study)

5.1 Introduction

The taxonomic importance of leaf epidermal surface characters and epicuticular wax as observed using SEM and TEM has been considered in many studies over the last half century (e.g. Barthlott, 1990; Barthlott *et al*., 1998). Stace (1965, 1984) and Wilkinson (1979) have also suggested that epidermal characters in the leaf, and other non-reproductive organs, are very useful characters in plant taxonomy. Several studies have been published which use epidermal characters within in the Apiaceae (Heywood, 1968, 1971). The number of epidermal cells, neighbouring and/or subsidiary, surrounding stomata (two cells versus three to five) have been shown to be important characters and used in the definition of many genera, subgenera or sections (Ostroumova & Kljuykov, 2007). Stomata types of the Apiaceae have proved to be of some taxonomic value (e.g. Guyot 1965, 1966, 1971; Ostroumova, 1987, 1990; Ostroumova & Kljuykov, 1991). Metcalfe and Chalk (1979) recorded paracytic and anomocytic stomata type in various genera of the Umbelliferae. Gupta, Paliwal and Manju (1965) reported anisocytic, paracytic and anomocytic stomata in *Bupleurum tenue*. Guyot (1971), who studied more than 150 different species of Apiaceae, found a consistency within each genus for stomata form.

There are relatively few published accounts of *Heracleum* leaf micro-morphological studies using SEM. Guyot (1982) and Mandenova *et al*. (1982) found that the most frequent stomatal form was anomocytic, with the exceptional

occurrence of paracytic, dicytic and mesogenous anisocytic stomata. *Heracleum sphondylium* (from France) and *H. mantegazzianum* were reported as possessing anomocytic stomata. In the comparative epidermal study of *H. mantegazzianum*, *H. sphondylium* and their hybrids, Arora *et al*. (1982) reported that both anomocytic and anisocytic stomata occur in each species, as well as tetracytic stomata, a form not recorded for *Heracleum* by Guyot (1982). Zarinkamar & Jalili (2004) demonstrated that in two Iranian species of *Heracleum* (*H. pastinacifolium*, *H. persicum*) anomocytic stomata are more frequent than anisocytic. Recently, in a broad survey of stomatal types in Chinese and Himalayan Umbelliferae, Ostroumova & Kljuykov (2007) reported that among 13 *Heracleum* taxa (10 species; 5 taxa from India, 8 taxa from W China) the anomocytic stomatal type is most frequent, with the concurrence of hemiparacytic type. However, the majority of Chinese and Himalayan *Heracleum* species (about 40 spp.) stomatal characters have not been investigated fully, and increased sampling for this species is needed to complete the global picture.

Compared to fruit surface studies there are few reports of trichomes on the leaf surface. Guyot (1982) investigated trichomes in the Apiaceae to assess their taxonomic value. From his study of the tribe Caucalideae, he concluded that they were taxonomically useful and that the shape of the hair apex and the ornamentation of the surface were more reliable characters than the density and length of hairs. In a comparison of 20 species of *Heracleum* which mainly come from Himalaya and India, Guyot (1982) came to the conclusion that it was not possible to subdivide the genus on the basis of leaf surface characters alone. In particular, he commented on the variability of ornamentation and suggests it to be

a character of secondary importance to apex form. In contrast, by a careful description, Aroral *et al*. (1982) showed that the length, shape and ornamentation patterns on the hair surface were sufficient to distinguish between *Heracleum sphondylium*, *H. mantegazzianum* and their hybrids.

This chapter reports finding of a pilot survey on the features of the epidermis, to assess the value of these past characters for Sino-Himalayan *Heracleum* and to see if other attributes could be found which might be useful in sub-generic or infra-generic classification.

5.2 Material and Methods

5.2.1 Material

Dried herbarium material was used to study leaf epidermis. Specimens were carefully selected with no treatments of specimens which can cause effect of wax patterns. Detailed voucher information is given in below (Table 5.1). At least 1 species (plant) per 3 regions (parts) are sampled.

5.2.2 SEM

Scanning electron microscope (SEM) examinations were made on herbarium leaf material. The specimens were first examined under a stereo microscope. Selected samples were transferred by fine forceps to 12mm carbon discs mounted on 12.5 mm aluminium pin SEM stubs. The material was splutter coated with gold palladium using a K575x sputter coater (Emitech) at a rate of 25mA for

2.5 minutes. The samples were then studied under a LEO supra 55VP digital scanning electron microscope. Stubs were first scanned at low magnification to select suitable material. The working distance was between 9 and 12 mm and the scanning voltage (EHT) set to 5 kV. Selected materials were then saved at different magnifications. Images were stored on CD-ROM and also printed out as video prints.

Table 5.1 Voucher information of leaf epidermis observation using SEM

Species	Source and Voucher
Heracleum bivitatum H. Boissieu	China, Yunnan, *s.n* (E)
H. biternatum W. Smith	Burma, upper Burma, Kingdon-ward 18864 (BM)
H. burmanicum Kurz	Thailand, Chiang Mai, 2250m, Beusekom & Phengklai 2387 (E)
H. bhutanicum M.F. Watson	Bhutan, Watson 6811 (E)
H. candicans Lindley	China, Yunnan, Dêqên Zang Aut., 3450 m, RBGE exp. 264 (E)
H. canescens Lindley	Nepal, Jumla, Polunin *et al.* 4461 (E)
H. franchettii M. Hiroe	China, Qinghai, Nangqên Xian, Ho *et al.* 2633 (BM)
H. kingdonii H. Wolff	China, Yunnan, Zhongdian, haba, 4000 m, Ace 2137 (E)
H. lallii Norman	Nepal, Bhurchula Lekh, near Jumla, 12,500 fit, Polunin *et al.* 4581 (E)
H. lanatum Michx.	USA, Montana, Clements 6297a (E)
H. lanatum Michx.	Japan, Furuse 6386 (E)
H. millefolium Diels	China, Ho *et al*. 489 (E)
H. nepalense D. Don	India, Skkim, East district, Fambong Lho, 2080 m, Long & Noltie 21 (E)
H. obtusifolium DC.	China, Tibet, langong, Ludlow *et al.* 5508 (E)
H. pinnatum C.B. Clarke	India, Ludlow & Sheriff 8436 (E)
H. scabridum Franch.	China, Yunnan, Likiang, Rock 5631 (E)
H. stenopterum Diels	China, Yunnan, Cee 436 (E)
H. sublineare C.B. Clarke	Nepal, Bheding, 12000-13000ft, Dhwoj 272 (E)
H. wallichii DC.	Nepal, Malemchi, Stainton 6666 (BM)
H. woodii M.F. Watson	Bhutan, Thimpu District: Saga La to Dungsho La, 4200m, Wood 7572 (E)
Tordyliopsis brunonis DC.	Nepal, Sinclair & Long 463 (E)

5.3 Result and Discussion

Leaf surfaces of several representative species of *Heracleum* were investigated. The surfaces were seen to differ in hair characters, especially the type of hairs, the pattern of hair surface, size, density and distribution. Stomata also differed somewhat in shape, particularly the surrounding epidermal cells. Some differences in lamina cell types were also observed. Results are presented below according to: 1. Trichome type, and 2. Laminar cell type.

5.3.1 Trichome type and hair surface ornamentation

Several characteristics of trichomes were investigated and are summarized in Table 5. 2. Trichomes are found both on adaxial and abaxial surfaces of the leaf. The size, density and distribution of trichomes (on veins or lamina surface) were seen to be very variable and not taxonomically useful. However, trichome type is consistent with the following four different trichome types delimited, based on a combination of several characters: simple non-glandular trichomes (apex either round or narrow in shape, or long ribbon shaped) and multicellular elongate-headed trichomes:

Type 1: Simple non-glandular trichomes with round hair apex (Fig. 5.1:1) Cylindrical shaped, hollow trichome, relatively short compared to other types of trichomes. This trichome type was observed in *H. wallichii*, *H. burmanicum*, *H. lanatum*.

Type 2: Simple non-glandular trichomes with sharp hair apex (Fig. 5.1: 2-3) Conical, wholly flat and with a sharp hair apex. Various sizes were observed in *Heracleum canescens, H. franchetti*, *H. sublineare* and *H. stenopterum*.

Type 3: Simple non-glandular trichomes with a long ribbon shape (Fig. 5.1: 4)

A distinctive long ribbon shape, wholly flat and densely distributed. This trichome type was only observed in *H. obtusifolium* and *H. candicans*. These two species have been often mis-identified because of morphological similarity and overlapping distribution. Cannon (1978) commented that *H. obtusifolium* is probably only a minor variant of *H. candicans,* and this new character supports this view.

Type 4: Multicellular elongate-headed glandular trichomes (Fig. 5.1: 5-6) Cylindrical or conical and more or less flat trichomes with an elongate-glandular tip was only founded in *H*. (=*Tordyliopsis*) *brunonis.* This glandular hair type is here observed for the first time within the *Heracleum.* The presence of elongate-headed glandular trichome is very interesting, and this very distinctive trichome type seems to strongly support the separation of this species from other *Heracleum* species. It supports de Candolle's treatment (1830), where he described the monotypic genus *Tordyliopsis*.

In addition to differences in trichome type, various ornamentations of hair surface were also recognized. Five different ornamentations (epicuticular striation) of hair surface are recognised: practically smooth type, horizontally striate type, densely papillose type, striate with papillose type, and vertically striate type.

Type 1: Smooth trichome surface (Fig. 5.2: 1-2)
Type 2: Papillose trichome surface (Fig. 5.2: 3, 6)
Type 3: Striate with papillose trichome surface (Fig. 5.2: 2, 5)

Distinctive surface ornamentation patterns were found within the all the species investigated, and this was useful to distinguish species within *Heracleum* that had the same trichome type as defined above. From this study the shape of the hair apex and the ornamentation of the surface are seen to be reliable characters, and seem to be important taxonomic characters in characterising species within *Heracleum*.

5.3.2 Trichome density and distribution

Trichomes are found on both veins and lamina cells, except *H. canescens* (trichomes only found on veins). The density varied between moderate to dense on veins and sparse to very dense on lamina cells. But these characters did not correspond to the other characters.

5.3.3 Laminar cell type and stomata type

The shapes of epidermal cells were seen to be similar on both the adaxial and abaxial leaf surfaces. These mostly have an undulate/sinuate or polygonal anticlinal wall (see Fig. 5.3), but occasionally this is circular especially in *Heracleum sublineare*. The epicuticular wax (cuticular striae) are well developed in most of the studied taxa and have different orientations. The following three main different laminar cell type and epicuticular wax striation patterns were recognized:

Type 1: Polygonal shape of epidermal cells with parallel striae (Fig. 5.3: 1, 4, 6)
Type 2: Undulate/sinuate shape of epidermal cells with centre oriented cuticular striae (Fig. 5.3: 5)
Type 3: Circular shape of cell with centre oriented cuticular striae (Fig. 5.3: 2, 3)

Within the all species investigated type 1 (polygonal shape, parallel striation) is most common. Distinct laminar cell type was found *in H. sublinear* and *H. wallichii*. These two species delimitation has sometimes been confused because of morphological similarity (long and narrow leaflets) and overlapping geographical

distribution. However, this study shows that *H. sublineare* has circular shape of cell with centre oriented cuticular striae but *H. wallichii* has strongly undulate/sinuate shape of epidermal cells with centre oriented cuticular striae. So this new finding seems to be good character for delimitation of species.

Among the stomatal type, an anomocytic stomatal arrangement, with the guard cells surrounded by more than three randomly placed cells, was the most commonly observed (Fig. 5.4: 1-2, 4-5). Stomata with tetracytic (guard cells surrounded by four subsidiary cells in a cruciform arrangement (Fig. 5.4: 3, 6) were also observed. The proportions of these kinds of stomata differ between the taxa. On the surrounding epidermal cells, well developed cuticular striations were visible. Because of this, during SEM survey of leaf stomata it is sometimes difficult to recognize the exact shape of the stomata complex. For reliable identification and elucidation of stomatal study, more detailed investigations (e.g. leaf-peel method) will be required for the future study.

5.4 Conclusion

In this pilot study of leaf micro-morphology in Sino-Himalayan *Heracleum*, several epidermal characters (trichome type, the shape of epidermal cells, and the stomata characters) were seen to be of taxonomic importance in differentiating some species. In particular *Tordyliopsis brunonis* has a unique trichome structure

with apical glands. *Heracelum obtusifolium* and *H. candicans* have long, ribbon-shaped trichomes that give a lanate texture to the abaxial surface of the leaves and *Heracleum franchetii* was the only species observed that had tetracytic stomata. Furthermore, these preliminary investigations suggest that there are some useful micro-morpholgoical characters that could be used for grouping species, but only the ribbon-shaped trichomes in *Heracelum obtusifolium* and *H. candicans* suggest relationships as synapomorphies.

Although the groupings looked promising to start with, once the molecular work had been done there was little congruence with the molecular groups (See chapter 8), which indicated that the characters may be homoplastic. The general function of trichomes and leaf epidermal cells are known for protection of UV, herbivores (insects), and very dry conditions (Metcalfe & Chalk, 1983). Therefore, various ecological factors (high altitude, strong UV, etc.) may effect convergence of leaf micro-morphology architecture as adaptations to specific environments may have resulted in many no-monophyletic groupings.

As this was a pilot study, only one of each species was investigated, and although it showed some interesting results for characterising individual or a couple of species pairs, because of the inconsistency across the species the characters were not very useful in creating infra-generic groups. For this reason, this pilot study was discontinued in favour of fruit morphological investigation.

Table 5.2 Leaf epidermis observations of selected species of *Heracleum*

Trichome type: 1: simple unicellular & round hair apex, 2: simple unicellular & sharp hair apex, 3: simple & long ribbon shape, 4: elongate-headed glandular.
Trichome surface: 1: smooth, 2: papillose, 3: striate.
Trichome density: 1: very dense, 2: dense, 3: moderate, 4: sparse; d: adaxial, b: abaxial
Trichome distribution: 1: vein only, 2: vein & lamina cells.
Cuticular striation of lamina cells: 1: parallel striation, 2: undulate striation, 3: central striation.
Stomata shape: 1: anomocytic, 2: tetracytic; ?: not certain.

Species	**Trichome**				**Lamina**	**Stomata**
List	**Type**	**Surface**	**Density (vein/lam.)**	**Distribution**	**Striation**	**Shape**
H. wallichii	1	3	3/4(d), 3/4(b)	2	3	1
H. burmanicum	1	2	3/3(d), 3/4(b)	2	1	1
H. obtusifolium	3	1	3/4(d), 1/1(b)	2	?	?
H. candicans	3	1	3/4(d), 1/1(b)	2	1	?
H. bivittatum	3	1, 3	3/3(d), 1/3(b)	2	2	1
T. brunonis	4	1, 3	2/4(d), 2/3(b)	2	1	1
H. franchetii	2	2	3/3(d), 2/2(b).	2	1	3
H. stenopterum	2	1, 3	3/3(d), 2/2(b)	2	1	1
H. kingdonii	1	2	3/?(d), 3/?(b)		2	1
H. nepalense	1	2	3/4(b), ?	1	?	?
H. lallii	2	1	3/4(d), 3/4(b)	2	2	1
H. woodii	1	1	3/4(d), 3/3(b)	2	1	1
H. sublineare	2	1	3/4(d), 3/4(b)	2	3	1
H. lanatum	1	2	3/2(d), 3/2(b)	2	?	1
H. canescens	2	1	3/4(d), 3/4(b)	2	2	1

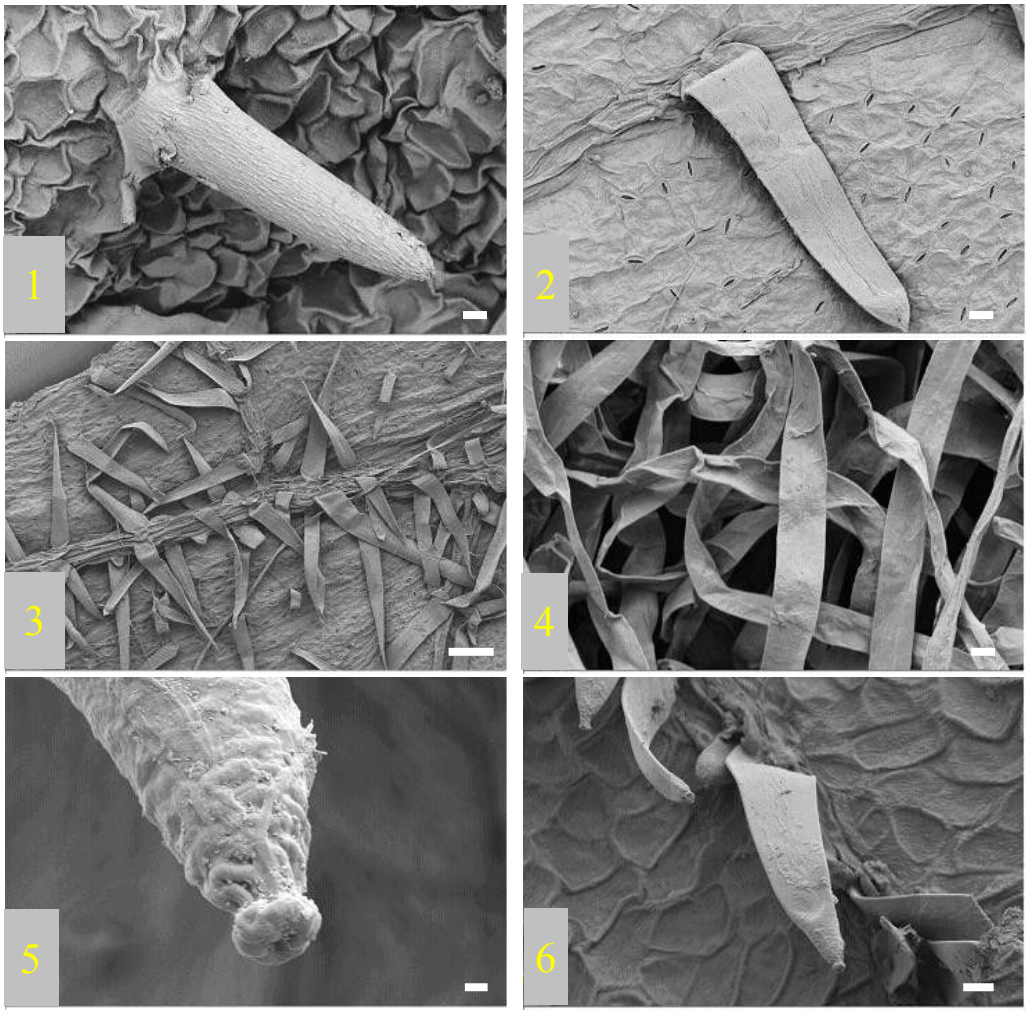


Fig. 5.1 SEM micrographs of trichomes in *Heracleum*.

1: *Heracleum woodii* M.F.Watson (Wood 7572), **2**: *H. canescens* Lindley (Polunin *et al.* 4461), **3**: *H. stenopterum* Diels (Cee 436), **4**: *H. obtusifolium* DC. (Ludlow *et al.* 5508), **5-6**: *H.* (=*Tordyliopsis*) *brunonis* DC. (Sinclair & Long 463). Scale bars: 1, 2, 6 = 20 um; 3 = 100 um; 4 = 10 um, 5 = 2 um.

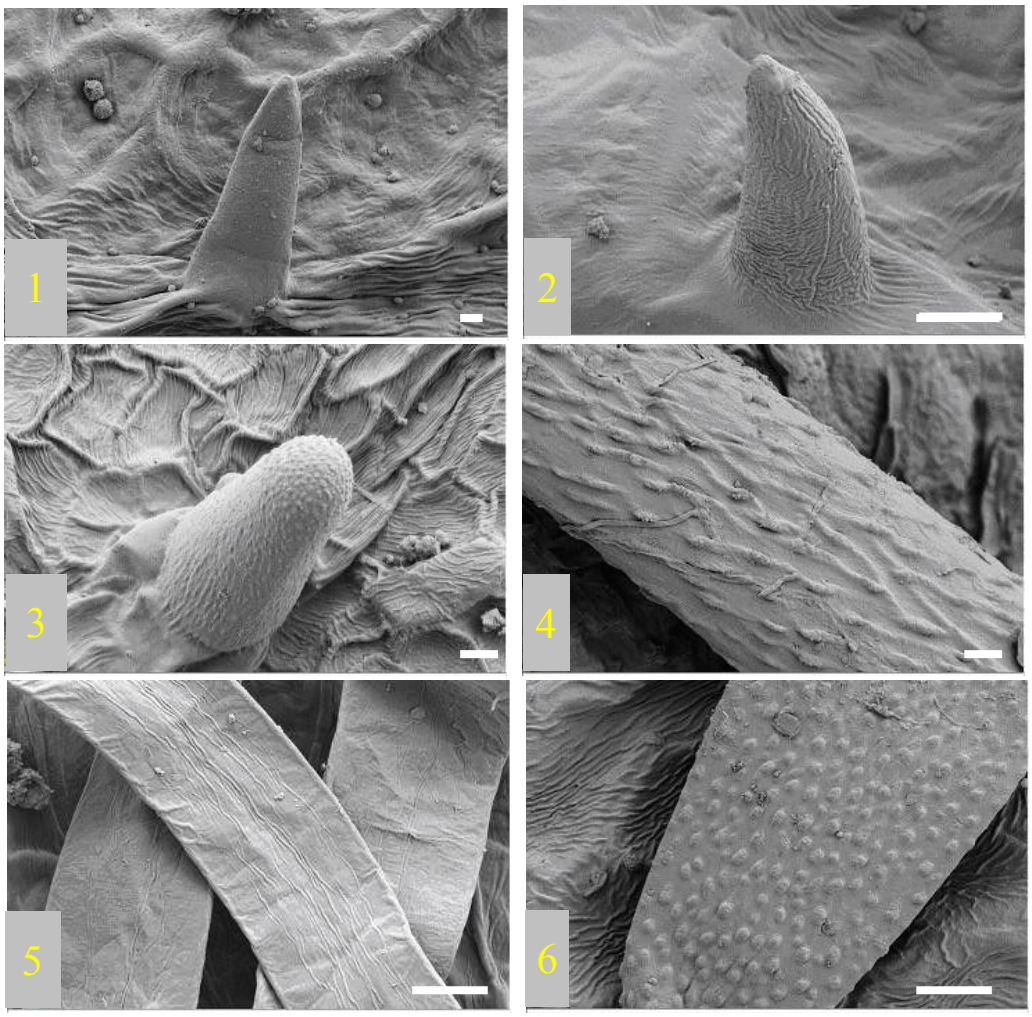


Fig. 5.2 SEM micrographs of various ornamentations of trichome surface in *Heracleum*.
1: *Heracleum bivittatum* H.Boissieu (*s.n*), **2**: *H. pinnatum* C. B. Clarke (Ludlow & Sheriff 8436), **3**: *H. burmanicum* Kurz (Beusekom & Phengklai 2387), **4**: *H. bhutanicum* M.F. Watson (Watson 6811), **5**: *H. bivittatum* H.Boissieu (*s.n*), **6**: *H. biternatum* W. Smith (Kingdon-ward 18864). Scale bars: 1, 4, 5 = 10 um; 2, 3, 6 = 20 um.

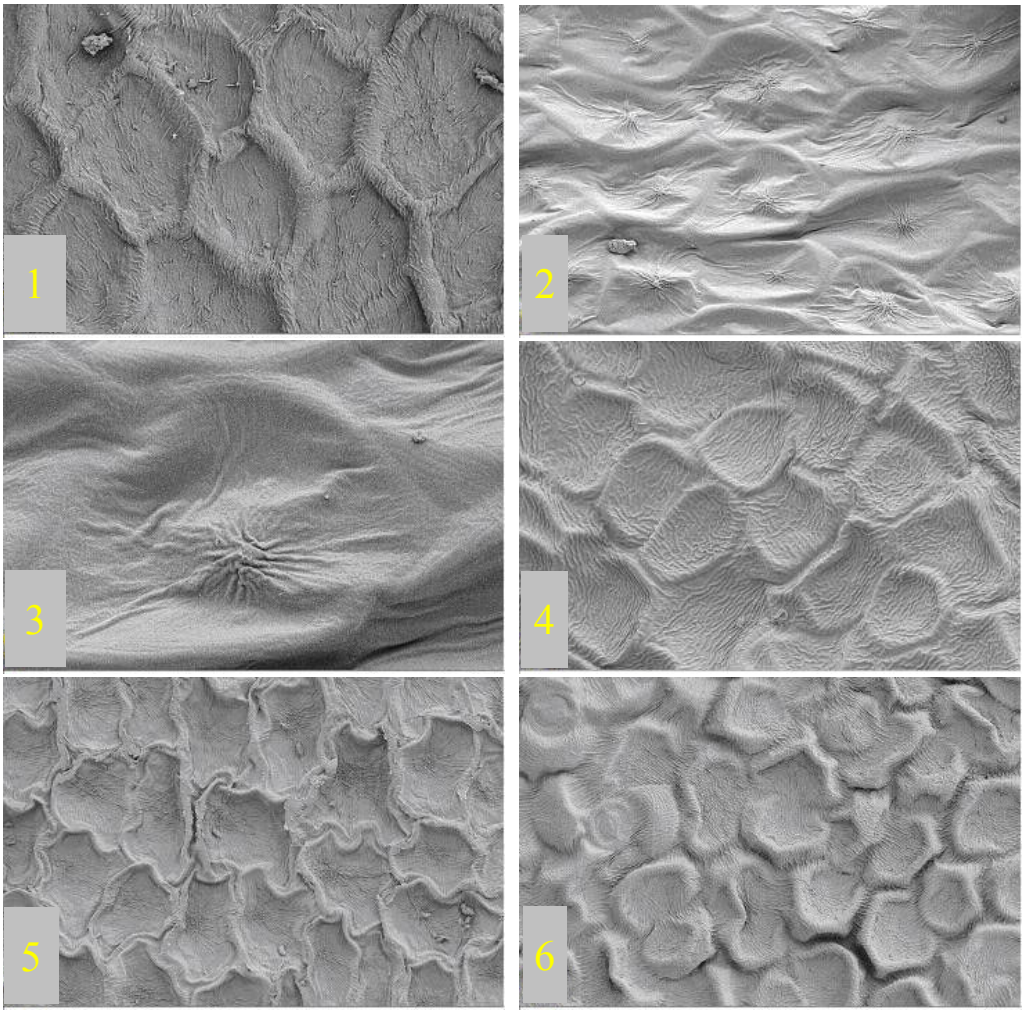


Fig. 5.3 SEM micrographs of leaf epidermis of *Heracleum*.

1: *H. canescens* Lindley (Polunin *et al.* 4461), **2-3**: *H. sublineare* C.B. Clarke (Dhwoj 272), **4**: *H*. (=*Tordyliopsis*) *brunonis* DC. (Sinclair & Long 463), **5**: *H. wallichii* DC. (Stainton 6666), **6**: *H. scabridum* Franch.(Rock 5631). Scale bars: 1, 2, 3, 6 = 10 um; 4, 5 = 20 um.

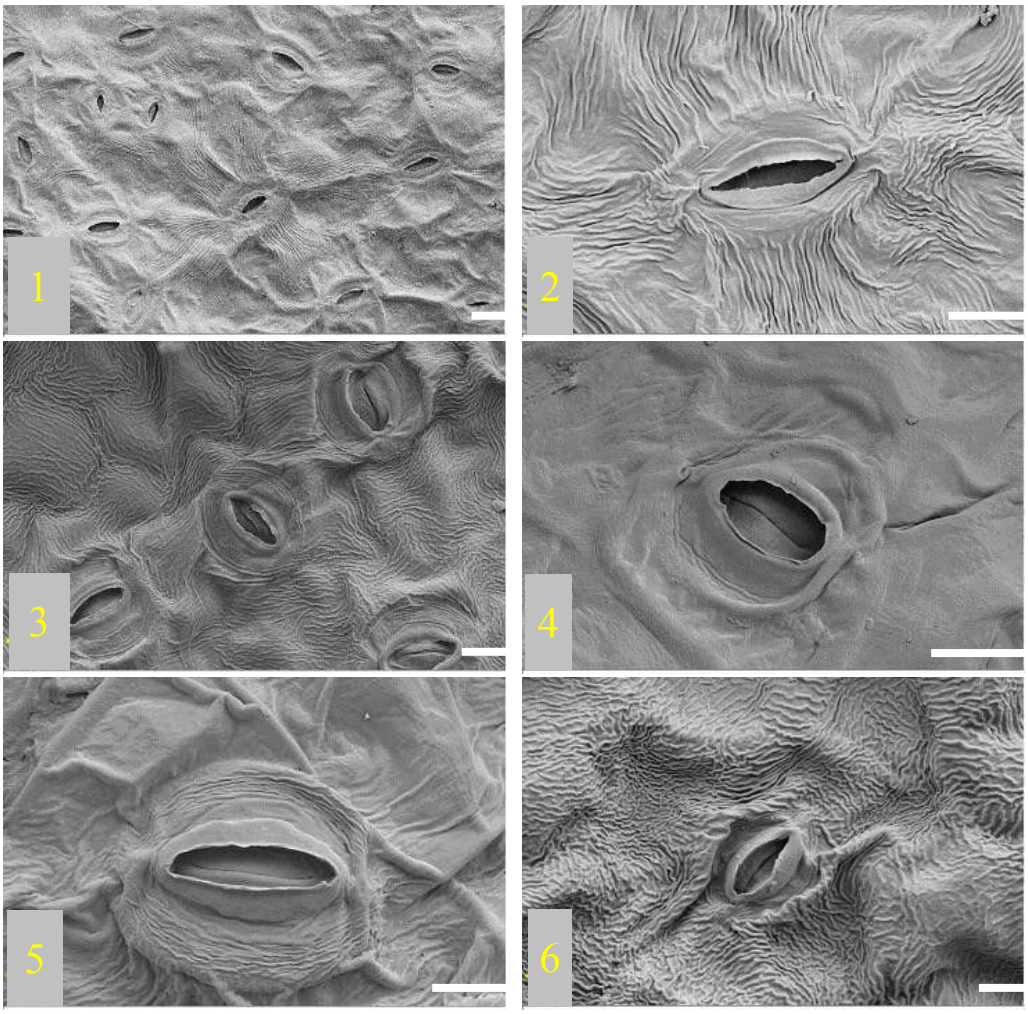


Fig. 5.4 SEM micrographs of leaf epidermis of *Heracleum*.

1: *H. burmanicum* Kurz (Beusekom & Phengklai 2387), **2**: *H. canescens* Lindley (Polunin *et al.* 4461), **3**: *H. franchetii* M.Hiroe (Ho *et al.* 2633), **4**: *H. biternatum* W. Smith (Kingdon-ward 18864), **5**: *H.* (=*Tordyliopsis*) *brunonis* DC. (Sinclair & Long 463), **6**: *H. franchetii* M.Hiroe (Ho *et al.* 2633). Scale bars: 1 = 20 um; 2 - 6 = 10 um.

Chapter 6: Fruit Anatomy

6.1. Introduction

Anatomical studies have played a very significant part in the historical development of our present-day concepts of relationships within the Apiaceae (Theobald, 1971). Fruit morphological characters have traditionally been used in tribal and generic delimitation within this family. For instance, as early as 1672, Morison apparently made use of fruit sections in describing his taxa. Subsequent investigators, including Crantz (1767), Hoffmann (1816), Koch (1824), and de Candolle (1830) all utilized similar gross anatomical features of the fruit in their investigations. Many past systems of Apiaceae classification, including the most influential system of Drude (1898), are also usually based on fruit characters, particularly its shape and compression, the characteristics of ribs, secretory canals, indumentum, endocarp sclerification, shape of endosperm, and the distribution of calcium oxalate crystals (Koch, 1824; de Candolle, 1830; Bentham, 1867; Drude, 1898; Calestani, 1905; Koso-Poljansky, 1916).

For the genus *Heracleum*, there have been several studies on the anatomy of the fruits (Briquet, 1924; Mandenova, 1950; Kowal, 1975), most on European members and a few near-Asian (Caucasus region) members of the genus. These fruit characters have often been used in infrageneric classifications into sections (de Candolle 1830; Mandenova 1950, 1962, 1982, and 1987; Satzyperova 1984; Tamamschjan 1967). However, until now, Sino-Himalayan *Heracleum* have not been studied in detail for their fruit anatomy, but rather much emphasis has been

placed on the superficial fruit characters such as rib form, outline shape and vittae number and distribution (de Candolle, 1830; Bentham, 1867; Clarke, 1879; He *et al*., 1998).

Related genera such as *Tordylium*, *Malabaila*, and *Zosima* have often been grouped together with *Heracleum* the tribal (Koch, 1824; de Candolle, 1830; Bentham, 1867) or sub-tribal level (Drude, 1898) based on the gross morphology of fruit. Lai's (1971) pilot study using comparative fruit anatomy indicated that there is a great deal of similarity between these genera. Lai also confirmed the consistencies within the subtribe Tordyliinae (Drude, 1898). Since then, there have been several investigations on the anatomy of fruit surface and transverse section for these closely related genera, for example *Malabaila* (Pimenov & Ostroumova, 1994), *Pastinaca (*Menemen & Jury, 2001a), *Tordylium* (Al-Eisawi & Jury, 1988), *Tordyliopsis* (Pimenov *et al*., 2000), and *Zosima* (Menemen & Jury, 2001b). These studies have given useful characters for delimitation of genera and subgeneric divisions down to the species level. However, *Heracleum* itself, particularly Sino-Himalayan *Heracleum*, has not yet received great attention from detailed anatomical study. Therefore, it was expected that the fruit anatomical investigation would provide characters of good taxonomic value for these Sino-Himalayan *Heracleum* species, and so a detailed study of fruit anatomy was undertaken to fill these knowledge gaps. These characters were also evaluated as to their usefulness at the generic or even tribal level, especially in comparison with related allies of *Heracleum*.

Downie and collaborators' molecular studies on the phylogeny of the Apiodeae (Katz-Downie *et al*., 1999; Downie *et al*., 2000a & b; Downie *et al*., 2001, and see Chapter 8) have recognised a '*Heracleum* clade' that remains stable in many of the studies. The '*Heracleum* clade' is currently known to contain *Heracleum*, *Pastinaca*, *Malabaila*, *Tetrataenium*, *Zosima* and *Tordylium*. The present study conducts an in-depth anatomical investigation of this '*Heracleum* clade', including accessions of many allied genera not yet formally included in molecular studies (such as *Semenovia*, *Tordyliopsis*) in order to determine the relationships among the '*Heracleum* clade' more fully.

6.2 Material and methods

6.2.1 Material

Dried herbarium material was used to study fruit anatomy. At least two mature fruits of each species were studied. Detailed voucher information is given in Table 6.1

Table 6.1 Voucher specimens for fruit anatomical study of Sino-Himalayan *Heracleum* and related genera.

Species	Source and Voucher
Heracleum bhutanicum M.F. Watson	Bhutan, Chukka District, Chuka, 1400m, Wood 6811(E)
H. burmanicum Kurz	India, Moflong, Khasia hills, Hooker 1974 (K)
H. burmanicum Kurz	Thailand, Winet 1800 (K)
H. candicans DC.	China, Sichuan, Muli, Yü 14153 (E)
H. candicans DC.	China, Yunnan, Děqěn, RBGE exp. 264 (E)
H. candolleanum (Wight & Arn.) Gamble	India, Tamil, Anamallay forest, White *s.n.* (K)
H. canescens Lindley	India, Gamble 5710A (K)

H. canesens Lindley	India, Simla, Mahasu road, Gamble *s.n*. (K)
H. franchetii M. Hiroe	China, Qinghai, Ho *et al*. 2633 (BM)
H. franchetti M. Hiroe	China, Qinhai, Chindu, Ho *et al.* 1752 (BM)
H. hemsleyanum Diels	China, Jiangxi, Yao 8669 (P)
H. kingdonii H. Wolff	China, Yunnan, Zhongdian, Ace 2137 (E)
H. lanatum Michx.	Japan, Furuse 6641 (K)
H. millefolium Diels	China, Qinghai, RBGE exp. 78 (E)
H. nepalense D. Don	India, Sikkim, Fambong Lho, Long & Noltie 21 (E)
H. obtusifolium DC.	Qinghai, yushu Xian, 3600 m, Ho *et al.* 2339 (BM)
H. pinnatum C.B. Clarke	India, Hooker *s.n.* (K)
H. rigens Wall.	India, Wight 1192 (K)
H. souliei H. Boissieu	China, Sichuan, Wang 1317 (PE)
H. sphondylium L.	United Kingdom, Brumitt 19044 (K)
Malabaila secacul (Banks & Sol.) Boiss.	Flora of Persia, Azerbaijan, Lamond 3833 (E)
Pastinaca sativa L.	Belgique, Couvin, Auquier 1694 (E)
Semenovia transiliensis Regel & Herd.	Russia(C Siberia), Roldugin *s.n.* (NY)
Tordyliopsis brunonis DC.	India, Himachal Pradesh, McBeath 2221 (E)
Tordyliopsis brunonis DC.	India, Skkim, Hooker *s.n*. (K)
Tordylium aegyptiacum Lam.	Cyprus, Lamond & McClintock 2775 (E)
Zosima absinthifolia Link	Flora of Persia, Azerbaijan, Lamond 3822 (E)

6.2.2 Anatomy

Dried fruit samples from herbarium specimens were fully re-hydrated for the reconstruction of fruit. After the fruits are fully hydrated the water was removed and the fruit embedded in Glycol methacrylate (GMA; 2-hydroxyethyl methacrylate, HEMA), following the method of Feder & O'Brien (1968). These were then sectioned on a Leica ultra microtome (RM2235). Various parameters of the Feder &O'Brien's method were altered to optimise the method for *Heracleum*. Details of the optimised procedures are as follow:

Preparation of Fruit:

Mature fruits were collected from good quality herbarium collections, and reconstituted with mixture of 10% aqueous bis (2-ethylhexil) sulfosuccinate sodium salt and 98% acetone (6:1) for 24 hours at 40°C.

Dehydration:

After washing with distilled water the materials were pre-sectioned (the base of the fruit) to facilitate the dehydration and infiltration procedure.

- The samples were dehydrated slowly with the following ethanol series: 70% (3hrs), 90 % (6hrs), 95 % (12hrs), 100 % (24hrs), 100 % (24hrs).

Infiltration: Technovit 7100 products

Technovit 7100, based on hydroxyethyl-methacrylate (HEMA), Kulzer

- The material was embedded by using 100% ethanol + infiltration medium (100ml of Technovit 7100 base liquid + 1g of Technovit Hardener I) in the following sequences {5:1 (24hrs), 3:1 (24hrs), and 2:3 (24hrs), 1:5 (24-48 hrs)}.
- During the each step air bubbles were removed by a vacuum pump and samples were occasionally shaken.

Embedding:

The histoform S embedding mould allows 10 objects to be embedded at one time.

- As soon as the embedding medium (infiltration medium 11 ml + hardener II 1 ml) are mixed, the fluid starts hardening.
- Using a plastic pipet, the embedding medium is put into the holes in the histoform. The wells need to be completely filled so that the fluid surface is convex.
- Then use a pair of tweezers to put the fruit into centre of the embedding medium in the holes in the histoform.
- After several minutes the matrix is firm enough so that the fruit can be oriented so as their longest side is parallel to the side of the well.
- Leave the embedding medium to harden for at least a further 30 minutes, then place the embedding mould on a hot plate at 60 °C for 1-2 hours to fully polymerise.
- After embedding, place the histoform S embedding mould in a refrigerator (5°C) overnight, and then leave it at room temperature for 1 or 2 days

Mounting:

- The embedded specimens in the Histoform S mould are mounted using Technovit 3040 powder (20 ml) and fluid (10 ml) for ten holes.
- Dry the surface of the embedded objects using paper
- Label the histoblocks and place them in the recesses in the Histoform S mould.

- Prepare the mounting mixture in a glass vial, but as soon as they are mixed it starts to harden, so one has to work fast.
- Pour the mounting mixture into the recess at the back of the histoblocks to a level of about 2 mm above the base of the histoblock.
- The histoblock can be removed from the Histoform S mould after 20 minutes

Trimming:

- Put the histoblocks on a hot plate 60°C for 5 minutes to soften the block.
- Use razor blades to cut of the excess embedding material, and cut the object in the form of a polygon (trapezoid).

Cutting:

- Clamp the histoblock into the object holder on the microtome, making sure that the first contact between knife and object occurs at a sharp angle.
- Section (4 -7 µm) and check the sections using a binocular microscope (Leica DME); correct the position of the object

Staining:

- Mounted sections were stained using the Toluidine Blue (0.02% solution of toluidinblue) (0.05 g/250 ml aq. dest.)
- Stain the section for 1-3 minutes by placing a drop of solution on the section.
- Rinse in running water until the plastic embedding matrix is nearly stain-free.

Photography:

- Fruit structure was observed and photographed using a Zeiss semi 2000C and Zeiss Axiocam MRc5 Digital camera.

Table 6.2 Comparison of transverse section of mericarps with detailed hypendorcarp fibres orientation in *Heracleum* clade and related genera. dor: dorsal face, com: commissural face, T= Vertical fibres of hypendocarp, L=Horizontal fibres of hypendocarp.

Taxa	**Hypendorcarp arrangement**			
	Intercostal (dor) / (com)	**Around vittae (dor) / (com)**	**Seed cavity (dor) / (com)**	**Proximal part**
Heracleum candicans DC.	(4T) / (4T)	(1L) / (1L)	(3-4T) / (1-2L)	1L
H. obtusifolium DC.	(3-4T) / (4-5T)	(1L) / (2T)	(1-2L1-2T) / (2L1T)	1-2L
H. pinatum C.B. Clake	(4T) / (4T)	(2T-1T1L) / (2T)	(2-4T) / (2-3T1L)	?
H. canescence Lindley	(3-4T) / (4-5T)	(1L) / (1T)	(3T) / (4T)	2-3L
H. bhutanicum M.F. Watson	(3-4T) / (4-5T)	(1L) / (1T)	(3T) / (4T)	1-2 T
H. nepalense D. Don	(3-5T) / (4-5T)	(1L) / (1L)	(3-4T) / (5T)	1-2L
H. rigens Wall.	(3-4T) / (3-4T)	(1-2L) / (1T or 1L)	(2-3T1L) / (2-3T1L)	2-3L
H. kingdonii H. Wolff	(1T3L) / (3-5T)	(1T2L) / (1L)	(1T2L) / (3L)	1-2T3L
Tordyliopsis brunonis DC.	(3T1L-4T) / (2-4T2L)	(1T1L or 2L) / (1T1L)	(3T) / (4T)	3-4L
H. sphondylium L.	(2T2L) / (2T2LT)	(1T2L) / (2T1L)	(2T2-3L) / (1T3L)	1-2L
H. lanatum Michx.	(2T1L) / (3-4T)	(2T1L) / 2T1L)	(1T2-3L) / (3T2L)	1-2L
H. franchetti M. Hiroe	(3T2L) / (2-3T1-2L)	(3-4L) / (1-2L)	(2-3L) / (1T2L)	1T
H. souliei H. Boissieu	(1T2-3L) / (3T1L)	(2-3L) / (1L)	(3-4L1T) / (3-4L)	2T1L or 2L1T
H. hemsleyanum Diels	(1-2T2-3L) / (1T3-4L)	(3L) / 2L	(1-2T3L) / (3-4L)	4-5L
H. millefolium Diels	(2T2L-3T1L) / (3-4T1-2L)	(3-4L) / ?	(3L) / (2L1T)	3-4L
Pastinaca sativa L.	(3-4T2-3L) / (3T)	(1T3L) / (1L)	(3-4L) / (2-3L1T)	1T3L or 4L
Malabaila secacul (Banks & Sol.) Boiss.	(1T1-2L) / (3T-2T1L)	(2L-1T2L) / (1L)	(3-4L) / (1T2-3)	1-2L
Semenovia transiliensis Regel & Herd.	(1T3L) / (2-3T1-2L)	(2-3L) / (2-3L)	(4-5L) / (3-4L)	4-5L
Tordylium aegyptiacum Lam.	(3T/4L) / (3T2-3L)	(1-2T3-4L) / (1-2T3-4L)	(1T3-4L) / (3-4T)	4-5L / 2-3T
Zosima absinthifolia Link	-	(1L) / (1L)	(2-3L-1T) / (1L-3T)	-

Table 6.3 Comparison of anatomical structure of seed cavity and marginal ribs with mean value and standard deviations (STD) of mature fruit of *Heracleum* and related genera.

Taxa			Marginal ribs					
	Width of seed cavity		Width of proximal part		Width of distal inflated part		Thickness of distal inflated part	
	(mm)	(STD)	(mm)	(STD)	(mm)	(STD)	(mm)	(STD)
Heracleum candicans DC.	0.30-0.40	0.35±0.070711	0.38-0.51	0.44±0.091924	1.01-1.30	1.15±0.205061	0.19-0.24	0.21±0.035355
H. obtusifolium DC.	0.35-0.40	0.37±0.035355	0.60-0.82	0.71±0.155563	1.50-1.60	1.55±0.070711	0.17-0.19	0.18±0.014142
H. pinatum C.B. Clake	0.35-0.40	0.37±0.035355	0.34-0.35	0.34±0.007071	1.22-1.41	1.31±0.13435	0.20-0.23	0.21±0.021213
H. canescence Lindley	0.25-0.30	0.27±0.035355	1.28-1.46	1.37±0.127279	0.48-0.52	0.50±0.028284	0.10-0.10	0.10±0.00000
H. bhutanicum M.F. Watson	0.05-0.07	0.06±0.014142	0.89-1.02	0.95±0.091924	0.66-0.72	0.69±0.042426	0.08-0.12	0.10±0.028284
H. nepalense D. Don	0.18-0.20	0.19±0.014142	1.49-1.69	1.59±0.141421	1.15-1.20	1.17±0.035355	0.20-0.21	0.20±0.007071
H. rigens Wall.	0.50-0.60	0.55±0.070711	1.19-1.21	1.20±0.014142	0.78-0.92	0.85±0.098995	0.18-0.20	0.19±0.014142
H. kingdonii H. Wolff	0.40-0.60	0.50±0.141421	0.22-0.35	0.28±0.091924	0.54-0.64	0.59±0.070711	0.16-0.18	0.17±0.014142
Tordyliopsis brunonis DC.	0.45-0.55	0.50±0.070711	0.48-0.57	0.52±0.06364	0.60-0.73	0.66±0.091924	0.15-0.18	0.16±0.021213
H. sphondylium L.	0.62-0.63	0.62±0.007071	0.49-0.52	0.50±0.021213	0.75-0.80	0.77±0.035355	0.17-0.20	0.18±0.021213
H. lanatum Michx.	0.40-0.45	0.42±0.035355	0.61-0.57	0.59±0.028284	0.70-0.74	0.72±0.028284	0.21-0.25	0.23±0.028284
H. franchetti M. Hiroe	0.40-0.85	0.62±0.318198	0.19-0.27	0.23±0.056569	0.33-0.43	0.38±0.070711	0.18-0.24	0.21±0.042426
H. souliei H. Boissieu	0.50-0.55	0.52±0.035355	0.28-0.29	0.28±0.007071	0.41-0.54	0.47±0.091924	0.13-0.15	0.14±0.014142
H. hemsleyanum Diels	0.45-0.50	0.47±0.035355	0.39-0.59	0.49±0.141421	0.89-1.00	0.94±0.077782	0.22-0.26	0.24±0.028284
H. millefolium Diels	0.07-0.10	0.08±0.021213	0.53-0.72	0.30±0.323855	0.46-0.47	0.46±0.007071	0.14-0.16	0.15±0.014142
Pastinaca sativa L.	0.45-0.55	0.50±0.070711	0.13-0.17	0.15±0.028284	0.38-0.43	0.40±0.035355	0.15-0.21	0.18±0.042426
Malabaila secacul (Banks & Sol.) Boiss.	0.10-0.12	0.11±0.014142	1.04-1.14	1.09±0.070711	1.47-1.80	1.63±0.233345	0.32-0.34	0.33±0.014142
Semenovia transiliensis Regel & Herd.	0.17-0.20	0.18±0.021213	0.25-0.33	0.29±0.056569	0.52-0.54	0.53±0.014142	0.15-0.18	0.16±0.021213
Tordylium aegyptiacum Lam.	0.8-0.10	0.45±0.494975	0.83-0.87	0.85±0.028284	1.63-1.64	1.63±0.007071	0.45-0.56	0.50±0.077782
Zosima absinthifolia Link	0.14-0.26	0.20±0.084853	0.71-0.85	0.78±0.098995	1.20-1.58	1.39±0.268701	0.41-0.42	0.41±0.007071

6.3 Results

To investigate the taxonomic value of fruit characters in *Heracleum* and related genera, fruit morphological characters and mericarp anatomical characters (Table 6.2, 6.3) were recorded.

6.3.1 Fruit Morphology of *Heracleum* (Fig. 6.1, 6.2, 6.3, 6.4)

Fruits of *Heracleum* are typical umbelliferous schizocarps with two mericarps loosely connected at the carpophore, but easily detached from each other at maturity. The carpophore is 2-cleft to the base. Each mericarp is elliptic, obovoid, slightly obovate, or orbicular, and strongly dorsally compressed. The mericarp is glabrous to villous when mature. Each mericarp has 5 ribs evident: one dorsal, two lateral and two marginal. The marginal ribs form a broad wing, while the dorsal and lateral ones are slender. The vittae which are 1(-2) in each furrow, 2(-6) or absent on the commissure, are narrow, reaching to the base of mericarp or clavate and commonly shorter than the mericarp. The seeds are flattened dorsally with a concave face.

6.3.2 Fruit Anatomical characters of *Heracleum* (Fig. 6.5, 6.6, 6.7, 6.8)

In median transection the mericarp is broadly oblong to elliptic-oblong and strongly flattened dorsally. **The dorsal and lateral ribs** range from barely visible to prominent. **The marginal ribs** extend into the wing like outgrowths of the fruit and are usually straight or slightly recurved away from the commissure. These wings are morphologically thin and generally have a slightly thickened edge. In most species, **the epidermis** is glabrous, or it may have trichomes. **The epidermal cells** are approximately rectangular in transection with their long axis parallel to the surface, thin

walled and have a thin cuticle. **The mesocarp** is composed of 2-6 layers of parenchymatous cells and in most species these are 3-5 layered. The commissural face usually has fewer layers of cells because the cells collapse when the mericarps mature and separate. The inner layers of the mesocarp tend to become lignified with age and lignifications is usually strongest in those cells adjacent to the endocarp. The outer parenchymatous cells are large, oblong, and elongated periclinally. Most of the parenchymatous cells above the vascular bundle of the marginal rib wings are also lignified and pitted. No druses were evident. In some species, there is a small inconspicuous companion canal on the dorsal side of each bundle, but these are crushed as the fruits mature. Usually six large vittae are present in the mesocarp; four intercostals ones on the dorsal side and two along the commissural face.

Hypendocarp (inner fibrous mesocarp) commonly consists of 3-7 rows of lignified and pitted fibre-like sclereids. It consists of mixed number of horizontal fibres of hypendocarp (hfh) and vertical fibres of hypendocarp (vhf) or vhf dominantly comprised (*H. candicans*, *H. obtusifolium*, *H. pinnatum*, *H. bhutanicum*, *H. nepalense*, *H. rigens*). It is complete along the dorsal side and extends into the marginal vascular bundle at the base of the wing. On the commissural face one commonly finds gaps where the vittae or weakly lignified layers of cells are situated. In most species the innermost layer of endocarp consists of elongated and slightly lignified cells forming a layer which completely encloses the seeds. This has sometimes been interpreted as the ‘true’ endocarp and the lignified cells described above have been referred to the mesocarp. Bradley and Fell (1966) have described this as a ‘parquetry layer’ (Fig. 6.7).

6.3.3 Species descriptions of *Heracleum*

H. sphondylium **(Fig. 6.2, 6.9)**

The mericarps of are light-yellow, strongly compressed dorsally, elliptic or slightly obovate in outline, 7-8 mm long, and 4-5 mm wide, dorsal and commissural face sparsely pubescent. Three dorsal ribs (median and two lateral) are widely spaced or inconspicuous. Marginal ribs are winged, their proximal parts 0.4-0.5 mm long, distal part of marginal rib 0.7-0.8 mm long is slightly inflated, 0.15-0.20 mm thick. The excocarp adheres to the mesocarp, and has small, narrow rectangular cells. The exocarp covers the dorsal side, edges of marginal ribs and 1/6-1/5 of the commissural face of inflated part of marginal ribs. Outer mesocarp consists of several layers of thin-walled parenchyma collapsed at maturity. Inner mesocarp (hypendocarp) present, is composed of thick-walled lignified and pitted fibre-like sclereids, inner 1-3 layers of horizontal fibres of hypendocarp (= hfh), outer 1-3 layers of vertical fibres of hypendocarp (= vfh). Vascular bundles of dorsal ribs are situated in the middle of the ribs outer mesocarp, at maturity these fuse with the hypendocarp that is thickened under the vascular bundles. In the marginal ribs there are vascular bundles at the base of inflated part of the rib with very large sclerenchyma. Between the hypendocarp enclosing the seed cavity and sclerenchyma of marginal rib there are horizontal lignified fibres. The seed cavity is 0.5-0.6 mm wide. The proximal part is short and not distinguishable. The distal part of the inflated margin of the lateral rib consists of parenchyma cells with pitted walls, persistent at maturity. Dorsal vittae are solitary in each furrow, rather narrower than the furrows. Commissural vittae are two. The endocarp consists of thin-walled cells stretched horizontally, a layer which completely encloses the seeds.

The same basic fruit structure is also seen in *H. lanatum*, *H. franchetii*, *H. souliei*, *H. hemselyanum*.

H. lanatum

Mericarps are yellow, elliptic, slightly obovate, 9-10 mm long, 7.5-8 mm wide and sparsely pubescent on the dorsal and commissural sides. Dorsal vittae four, commissural vittae two. On the dorsal side hypendocarp consists of 1-3 layers of horizontal fibres and 1-3 layers of vertical ones, on commissural side 1-2 horizontal and 3-4 layers vertical fibres. Exocarp covers up to 1/5 of the inflated margin on the commissural face.

H. franchetii

Mericarps yellowish, glabrous on dorsal and commissural faces, orbicular, elliptic or broadly obovate, 6-7 mm long, 5.5-6 mm wide, strongly compressed dorsally. Three dorsal ribs are obsolete, filiform. Marginal ribs are broadly winged, their proximal part is thin, 1-2 mm long. on the dorsal side the hypendocarp consists of 3-4 layers of horizontal fibres and 1-3 layers of vertical ones. The inflated part of the marginal ribs consists of a large sclerenchyma mass , vascular bundles at the base and large persistent parenchyma cells with pitted walls in the most distal part. The exocarp covers 1/5-1/3 of the inflated margin of commissural face.

H. souliei

Mericarps yellowish, glabrous on dorsal and commissural faces, orbicular or elliptic, 8-9 mm long, 7-7.5 mm wide, strongly compressed dorsally. Three dorsal ribs are obsolete, filiform. Marginal ribs broadly winged, their proximal part is thin, 1-2 mm

long. Hypendocarp consists of 3-4 layers of horizontal fibres and 1-2 layers of vertical ones. The inflated part of the marginal ribs consists of a large sclerenchyma mass with vascular bundles at the base and large persistent parenchyma cells with pitted walls in the most distal part. The exocarp covers 1/5-1/3 of the inflated margin of commissural face.

H. hemsleyanum

Mericarps yellowish, glabrous on dorsal and commissural faces, broadly obovate, orbicular, 6-7 mm long, 5.5-6 mm wide, strongly compressed dorsally. Three dorsal ribs are obsolete, filiform. Marginal ribs are broadly winged, their proximal part is thin, 1-2 mm long. The histological structure of pericarp is similar to that of *H. sphondylium.* Hypendocarp consists of 1-3 layers of horizontal fibres and 1-2 layers of vertical ones. The inflated part of the marginal ribs consists of a large sclerenchyma mass with two vascular bundles at the base and large persistent parenchyma cells with pitted walls in the most distal part. The exocarp covers 1/6-1/3 of the inflated margin of commissural face.

Heracleum candicans **(Fig. 6.11: D-F)**

Mericarps yellowish, sparsely pubescent all over on the dorsal side and glabrous on commissural faces, elliptic or broadly obovate, 8-8.5 mm long, 5.5-6 mm wide, strongly compressed dorsally. Three dorsal ribs are situated close to each other, prominent (low-keeled). Marginal ribs are broadly winged, their proximal part is thin, 4-5 mm long. Distal part of rib is somewhat inflated 1.0-1.3 mm long, 2-2.5 mm thick. The histological structure of pericarp is similar to that of *H. sphondylium* group (Core-*Heracleum* species) but composition of the hypendocarp is different. Hypendocarp consists of 0-1 layers of

horizontal fibres and well developed 3-4 layers of vertical ones. The seed cavity is relatively narrow (0.3-0.4 mm). The proximal part is distinguishable and 1.01-1.30 mm long. The inflated part of the marginal rib consists of a large sclerenchyma mass with two vascular bundles at the base and large persistent parenchyma cells with pitted walls in the most distal part. The exocarp covers 1/10-1/4 on inflated margin of commissural face.

The same fruit structure has been observed in *H. obtusifolium*, *H. canescens*, *H. bhutanicum*, *H. nepalense*, *H. rigens.*

H. obtusifolium **(Fig. 6.11: A-C)**

Mericarp yellowish to brownish, obovate, slightly pubescent on the dorsal surface, glabrous on the commissural face, 11-12 mm long, 7.5-8 mm wide. Dorsal vittae usually four, equal and long or unequal, Commissural vittae two to four. Hypendocarp consists of 0-1 layers of horizontal fibres and of 3-4 layers of vertical fibres. The proximal part is thin and long.

H. canescens

Mericarp yellowish to brownish, obovate, slightly pubescent all over on the dorsal surface, glabrous on the commissural face, 10-12 mm long, 7-7.5 mm wide. Dorsal vittae usually four, equal and short or unequal. Commissural vittae two. Hypendocarp consists of 0-1 layers of horizontal fibres and of 3-4 layers of vertical fibres. The proximal part is thin and long

H. bhutanicum **(Fig. 6.10: A-D)**

Mericarp yellowish to brownish, orbicular, glabrous on dorsal and commissural faces, 6-7 mm long, 5.5-6 mm wide. Styles reflexed. Dorsal vittae usually four, equal and long or unequal, sometimes there are 1-2 additional short vittae. Commissural vittae two to four. Hypendocarp consists of 0-1 layers of horizontal fibres and of 3-4 layers of vertical fibres.

H. nepalense **(Fig. 6.10: E-H)**

Mericarp yellowish to brownish, elliptic, obovate, slightly pubescent on the dorsal surface, glabrous on the commissural face, 9-11 mm long, 7-8 mm wide. Dorsal ribs prominent, closely spaced. Vascular bundles of dorsal ribs are situated in top of the ribs in the outer mesocarp, and in contact with the hypendocarp that is well thickened under the vascular bundles. Dorsal vittae usually four, large, round, sometimes there are 1-2 additional short vittae. Commissural vittae two to four. Hypendocarp consists of 0-1 layers of horizontal fibres and of 3-5 layers of vertical fibres. The proximal part is thin and long, The inflated part of the marginal rib is long and narrow.

H. rigens

Mericarp yellowish to brownish, orbicular, glabrous, 5-9 mm long, 5-8 mm wide. Dorsal vittae usually four, equal and long or unequal, sometimes there are 1-2 additional short vittae. Commissural vittae two to four. Hypendocarp consists of mainly 3-4 layers of vertical fibres. The proximal part is thin and long., the inflated part of the marginal rib is long and narrow.

6.3.4 Species description of related genera

Pastinaca sativa **(Fig. 6.6, 6.12:A-C)**

Mericarps are yellowish to brownish, elliptic, 6-6.5 mm long, and 4-4.5 mm wide, glabrous on dorsal and commissural sides. Dorsal ribs are obsolete or slightly prominent (low-keeled), widely spaced. Vascular bundles of the dorsal ribs are situated in the middle of the ribs outer mesocarp, and in contact with hypendocarp that is well thickened under the vascular bundles. Dorsal vittae four, narrow, commissural vittae two. Exocarp cells with wide lumen and rather thin walls or with narrow tangentially stretched lumen and thick outer walls. The mesocarp consists of 3-4 rows of parenchymatous cells. Under the dorsal ribs vertical fibres of hypendocarp are well developed. Hypendocarp consists of 2-3 layers of horizontal and 1-4 layers vertical fibres. The proximal part is short, not distinguishable. Inflated distal part is very short. The exocarp covers 1/4-1/3 of the inflated margin of commissural face.

Malabaila secacul

Mericarps yellowish, glabrous on dorsal and commissural faces, orbicular or broadly obovate, 9-10 mm long, 7.5-8 mm wide, strongly compressed dorsally. Three dorsal ribs are situated close to each other, and are filiform. Marginal ribs are broadly winged, their proximal part is thin, distal part of rib is strongly inflated 1.4-1.8 mm long, 0.3-0.4 mm thick.

Heracleum millefolium **(Fig. 6.6: B, 6.13:A-D)**

Mericarps yellowish to brownish, orbicular, elliptic, 6.5-7 mm long, and 5.5-6 mm wide, sparsely pubescent all over the dorsal side, glabrous on commissural side. Dorsal ribs

obsolete, widely spaced, marginal ribs broader, winged, slightly inflated in distal parts. Vascular bundles of dorsal ribs are situated in middle of the ribs, and are inconspicuous. Large secretory ducts are present in some ribs. Dorsal vittae four, narrow, commissural ones two. The mesocarp consists of 3-4 rows of parenchymatous cells. Under the dorsal ribs vertical fibres of the hypendocarp are well developed. Hypendocarp consists of 1-3 layers of horizontal and 1-3 layers vertical fibres.

Tordyliopsis brunonis **(Fig. 6.6: C)**

Mericarps strongly compressed dorsally, orbicular, elliptic, 7-8 mm long, 5-5.5 mm wide, sparsely pubescent all over the dorsal side, glabrous on commissure side. Dorsal ribs obsolete, widely spaced, marginal ribs broader, winged, slightly inflated in distal parts. Commissural face wide. Exocarp consisting of one layer of small cells. Outer mesocarp layer of thin-walled parenchyma cells. Inner mesocarp (hypendocarp) composed of 1-2 layers of thick-walled horizontal lignified fibrous and 2-4 layers of vertical layers of fibres [the composition of the hypendocarp is similar to core-*Heracleum* group], Vascular bundles in the dorsal ribs are thin, in marginal ribs/wings they are more thick. Thin-walled, slightly lignified parenchymatous cells with pitted walls are present in the distal part of marginal ribs. The Proximal part is long. Inflated distal part is short and narrow [this proximal part and Inflated distal part is similar to that of the *Tetrataenium* group]. Exocarp covers 1/6-1/5 of the inflated margin of the commissural face. Dorsal vittae solitary in furrows, thin, extending for half or 2/3 of mericarp length, frequently unequal, commissural vittae absent or 1-2, short. Endocarp of hardly distinguishable cells. Endosperm plane on the commissural side.

***Zosima absinthifolia* (Fig. 6.6: E, 6.12:D-F)**

The mericarp is strongly compressed dorsally, orbicular, elliptic, 9-10 mm long, 7.5-8 mm wide, pubescent all over glabrous on commissure side. Dorsal ribs obsolete, marginal ribs, broader, winged, inflated distal parts are well developed. Commissural face is wide. Outer mesocarp 3-4 layer of thin-walled parenchyma cells. vittae solitary, large, almost occupying the entire areas of the mesocarp between each of the ribs. Inner mesocarp (hypendocarp) not uniform, poorly lignified, various numbers of horizontal and vertical fibres.

***Semenovia transiliensis* (Fig. 6.6: B, 6.13: E-H)**

Mericarps strongly compressed dorsally, elliptic, 6-6.5 mm long, 3.5-4 mm wide, sparsely pubescent all over glabrous on commissure side. Dorsal ribs low-keeled, narrowly spaced, marginal ribs broader, winged, slightly inflated in distal parts. Commissural face broad. Vittae solitary in furrows, large, elliptic. Exocarp consisting of one layer of small cells. Outer mesocarp layer of thin-walled parenchyma cells. Inner mesocarp (hypendocarp) mainly composed of 3-4 layers of thick-walled horizontal lignified fibrous. Vascular bundles in dorsal ribs thin, in marginal ribs/wings are more massive.

***Tordylium aegyptiacum* (Fig. 6.6: D)**

Mericarps orbicular or elliptic, strongly compressed dorsally, 8.5-9 mm long, 8-8.5 mm wide, vesicular hairs on the epidermis. Dorsal ribs obsolete, marginal ribs well developed, swollen. Outer mesocarp consists of 2-4 layers of parenchymatous cells. Inner mesocarp (hypendocarp) commonly consists of 3-5 rows of lignified fibre-like sclereids (1-3 layers of horizontal fibres and of 1-3 layers of vertical fibres). As in *Heracleum*, the

hypendocarp is complete along the dorsal side and extends to the marginal rib vascular bundles. It also extends through the marginal vascular bundle and reaches the edge of marginal wings

6.4 Discussion

6.4.1 Taxonomic value of anatomical characters

Among the characters mentioned in the above description not all the characters appear to have systematic value and some do not differentiate between taxa. A summary of features with potential taxonomic significance follows.

Seed cavity

Although the seed cavity size is variable, based on our results (Table 6.3) the general tendency was found that the core *Heracleum* group (*H. lanatum*, *H. franchetti*, *H. souliei*, *H. hemsleyanum*) have a relatively larger cavity size than the *Tetrataenium* group (*H. canescens*, *H. bhutanicum*, *H. nepalense*, *H. rigens*). In addition, the composition of the hypendocarp around the seed cavity of *Heracleum* group consists of mainly horizontal fibres, compared to the *Tetrataenium* group which has vertical fibres.

Proximal part size of the fruit

The proximal part is thin and consists of the combination of 1-5 layers of horizontal and vertical fibres. In all species examined width of the proximal part is variable in size (0.19 – 1.69 mm). However, general trends were found so that the width of proximal part can

be divided into two types (Table 6.3). In the first type, the proximal part is elongated (more than 0.83 mm: between 0.83 - 1.69mm) as in *H. canescens*, *H. bhutanicum*, *H. nepalense*, *H. rigens*, *H. kingdoniim*, *Malabaila secacul* and *Tordylium aegyptiacum*. In the second type, the proximal part is short (less than 0.83 mm: between 0.19 mm – 0.82 mm) as in *H. lanatum*, *H. franchetti*, *H. souliei*, *H. hemsleyanum*, *Pastinaca sativa*, *H. millefolium*, *Semenovia transiliensis*, *Tordyliopsis brunonis*, *H. candicans*, *H. obtusifolium* and *H. pinatum.*

Inflated distal part of the marginal rib

The inflated part of the marginal rib consists of a large sclerenchyma mass with 1-2 vascular bundles at the base and large persistent parenchyma cells with pitted walls. The shape is constant within each genus, but the size shows a small difference between closely related genera, and two types can be recognized (Table 6.3). In the first type the size of the inflated distal part is very long (more than 1.01 mm: between 1.01 – 1.80 mm), as in *H. candicans*, *H. obtusifolium*, *H. pinatum*, *H. nepalense*, *H. rigens*, *H. kingdonii*, *Malabaila secacul* and *Tordylium aegyptiacum*. In the second type, the size of the inflated distal part is short or relatively short (less than 1.00 mm: between 0.33 – 1.00 mm), as in *H. lanatum*, *H. franchetti*, *H. souliei*, *H. hemsleyanum*, *Pastinaca sativa*, *H. millefolium*, *Semenovia transiliensis* and *Tordyliopsis brunonis.*

The hypendocarp arragenment

The hypedocarp arrangement in the intercostals and around the seed cavity area can be divided into two types (Table 6.2). In the first the main composition of these area is vfh (vertical fibres of hypendocarp), as in *H. candicans, H. obtusifolium, H. pinatum, H.*

canescence, H. bhutanicum, H. nepalense, H. rigens, H. kingdonii and *H. brunonis.* In the second, the main composition of these area is hfh (horizontal fibres of hypendocarp) and/or vfh, as in *Heracleum lanatum, H. franchetti, H. souliei, H. hemsleyanum, Pastinaca sativa, Malabaila secacul, H. millefolium, Semenovia transiliensis* and *Tordylium aegyptiacum.*

6.4.2 The relationships among the '*Heracelum* clade'

Lai's investigation using anatomical and morphological comparison of flower and fruit (1971) concluded that there is a great deal of similarity between *Heracleum*, *Tordylium*, *Malabaila*, *Zosima* and *Pastinaca.* This comparison shows that these genera exhibit similar pattern of development and have several anatomical characters in common. The similarities include such anatomical characteristics as

(1) complete union of mericarp at anthesis
(2) lateral (=marginal: sensu Kljuykov *et al*., 2004) wings growing out together as a unit through development
(3) weak development of dorsal and intermediate ribs
(4) presence of a woody endocarp (= inner part of Mesocarp (hypendocarp))
(5) enlargement of the seed cavity

Lai's comparative anatomical study (1971) provided a fundamental basis for understanding the generic relationships in a subtribe Tordylieae (*Heracleum* and potentially related genera). In this study she noticed that there are several differences among the genera, such as the nature of wing margins and the number of fiber-like

sclereids around the marginal vascular bundle in the wing. However, she did not stress the differences between genera and did not treat the genus *Tetrataenium* as valid. Downie *et al*'s (2000a,b) very broad phylogeny of the Apioideae indicated that *Heracelum* is polyphyletic, and forms part of a broader '*Heracleum* clade' including accessions of many allied genera (*Semenovia*, *Tordyliopsis*). Our detailed anatomical survey not only confirmed Lai's study but also strongly supported '*Heracleum* clade' concept.

In the above discussion four taxonomically significant characters (width of seed cavity, width of the proximal part of the wing, width of the distal inflated part and hypendocarp arrangement) have been selected. Based on these characters, it can be hypothesised that apparent increase in fruit size is due to enlargement of the seed cavity and the extent of proximal part of which both part is caused by cell enlargement, and development of the distal inflated part.

According to this result, *Heracleum* can be divided into 2 main groups and the generic relationships with other related genera can be estimated. These groupings are strongly supported by ITS phylogenetic result (see chapter 8).

6.4.3 Heracleum *sensu lato* - relationships to allied genera

Group 1 (Core *Heracleum* group): All the species in this group exhibit a similar mode of anatomy and development. This includes *Heracleum lanatum, H. franchetti, H. souliei, H. hemsleyanum.*

- Most species of this group have short, thin wings and the portion of the endocarp between the seed cavity and marginal vascular bundle is short.
- The apparent increase in fruit size is due to enlargement of the seed cavity.
- The development of the seed cavity is caused by cell enlargement.
- Most species of the group have similar hypendorcarp composition in inter costal, around vittae and seed cavity area (1-2 layers of horizontal fibres and 1-2 layers of vertical fibres).
- The enlargement of proximal part is also caused by extention of 2-3 layers horizontal fibres.

Group 2 (*Tetrataenium* group): All the species in this group exhibit a similar node of anatomy and development. This group include *H. candicans, H. obtusifolium, H. pinatum, H. canescence, H. bhutanicum, H. nepalense, H. rigens.*

- Most species of this group have long, thin wings and the portion of the endocarp between the seed cavity and lateral vascular bundle is long.
- The apparent increase in fruit size is due to enlargement of proximal part and inflated distal part rather than seed cavity enlargement.
- The development of the proximal part is caused by extension of 2-3 layers horizontal fibres.
- Most species of the group have similar hypendorcarp composition in inter costal, around vittae and seed cavity area (0-1 layers of horizontal fibres and 3-5 layers of vertical fibres).
- Group 2 is formed from species that correspond to Mandenova's (1959, 1982) genus *Tetrataenium*, plus *H. bhutanicum* (a species Mandenova never encountered). This group is further supported by fruit morphological characters

(well-developed calyx teeth, better developed and narrowly carinate dorsal and intermediate fruit ribs, and elongate, truly claviform and septate dorsal vittae).

- Our morphological observations added further characters to this list; group 2 has 3-5 layers of vertical fibres in the hypendocarp, whilst the core *Heracleum* group has 1-2 layers of horizontal fibres and 1-2 layers of vertical fibres (Table 6.2).
- This evidence is treated in combination with molecular phylogenetic evidence (chapter 8) to resurrect the genus *Tetrataenium* for the treatment in the *Flora of Nepal*.

6.4.4 Related allies

Pastinaca and *Malabaila*: The histological structure of pericarp is similar to that of Core-*Heracleum* group. Only minor differences can be found (*Pastinaca sativa*: dorsal ribs low-keeled, vfh under dorsal vascular bundles is well developed and distal part is poorly developed than Core *Heracleum*; *Malabaila secacul*: Proximal part is thin and longer, distal part is long and well developed than Core-*Heracleum* group). *Pastinaca* (cultivated worldwide) and *Malabaila* (Mediterranean) characterized by yellow petals and simply pinnate leaves. Calestani's (1905) rather radical re-classification of these genera included *Malabaila*, *Heracleum*, *Zosima*, *Lophotaenia*, *Ainsworthia*, *Wendiana*, and *Tordylium* within *Pastinaca*. Later studies refuted this classification, and Menemen & Jury (2001a, b and c) suggested that these genera could be clearly separated from each other using characteristics of macro-morphology, micromorpholgy, anatomy, and palynology. Recent molecular studies (Yousef *et al*., In Press; Logacheva *et al*., In Press) strongly support the groupings of *Pastinaca* and *Malbaila* and the placement of clade close to the core *Heracleum* clade (See chapter 8). However even these molecular

results could not clarify the taxonomic relationships between species of *Pastinaca* and *Malabaila.*

Our anatomical study of mericaps support treating these genera as distinct, but with more extensive sampling beyond this two species is needed before any taxonomic relationships can be proposed.

- *Malabaila* and *Pastinaca* have a similar structure of inner endocarp.
- *Pastinaca* has slightly distinct (low-keeled) dorsal ridges.
- *Pastinaca* has poorly developed distal part', however, *Malabaila* has a much better developed proximal part and swollen distal inflated part.

Zosima absinthifolia has distinctive fruit anatomical characters such as large vittae, which completely fill the space between the two ribs, the linear leaf segments (Menemen & Jury, 2001c). The monophyly of *Zosima* is strongly supported with molecular data (99% of bootstrap value (see molecular chapter) and a distinctive composition of flavonols and flavones (Menemen *et al*., 1998).

- Large solitary vittae in the intervals and two on the commissure.
- The vittae almost occupy the entire areas of the mesocarp between each of the ribs. This is a very distinctive.
- Hypendocarp is not uniform, poorly lignified, various numbers of horizontal and verticlal fibres
- This group is strongly supported by bootstrap value 99% and distinctive composition of flavonols and flavones

Tordylium aegyptiacum - has typical anatomical characters of *Heracleum* s. l., except near the tip of the swollen wing margin and vesicular hairs on the epidermis. These are peculiar features of this genus. These distinctive characters are not observed in any other genera examined in the present study.

Tordyliopsis brunonis - has been treated as a *Heracleum brunonis* by past authors (Bentham & Hooker, 1867; C.B. Clarke, 1879; Drude, 1898). It was resurrected by I.P. Mandenova (1950, 1959, Mandenova *et al*., 1982). According to Mandenova (1959), *Tordyliopsis* differs from *Heracleum* by the presence of well-developed keeled dorsal mericarp ribs, septated vittae, and in petal shape, and differs from *Tetrataenium* in having large marginal ribs with a thin translucent proximal part and in its non-radiant marginal petals. Other authors regarded bract characters to support the separation of *Tordyliopsis* (Mukherjee & Constance, 1993; Watson, 1999 and 2005). Some fruit anatomical characters were noted by Mandenova (1982) and Mukherjee & Constance (1993). However, their descriptions of *Tordyliopsis* fruit were incomplete and partly contradictory (Pimenov *et al*., 2000). However, Our results show that dorsal ribs (obsolete, widely distributed) and hypendocarp structure (1-2 layers of thick-walled horizontal lignified fibrous and 2-4 layers of vertical layers of fibres) is similar to *Heracleum*, but the proximal part features (thin, long) are rather similar to *Tetrataenium* group. In this respect *Tetrataenium* may represent an intermediate.

Heracleum millefolium - The taxonomic position of *Heracleum millefolium* has long been problematic. This taxon was treated within *Semenovia* (Watson, 1999) or *Heracleum* (Pu & Watson, 2005) based on various vegetative or floral characters. Our result shows that *H. millefolium* has unique large rib secretory ducts, which are not present in *Heracleum*

or *Semenovia*. Because other anatomical characters are constant with other species, the taxonomic position of *H. millefolium* is still uncertain, but at least its separation from all *Heracleum* species sampled (and the other allied genera sampled, including *Semenovia*) is supported. Further sampling of *Semenovia* and related genera will help to confirm its position.

Semenovia - the delimitation of this genus has also been difficult. This central Asiatic genus (SW, S, Central Asia) comprising around 18 species, sometimes included within and sometimes separated from *Heracleum* (Pu & Watson, 2005; Watson, 1999). In our result *Semenovia transiliensis* has part of common characters of *Tetrataenium* group (dorsal ribs low-keeled, narrowly distributed) although the hypendocarp is mainly composed of 3-4 layers of horizontal lignified fibrous. Hence, these features support its separation from *Heracleum millefolium.* On the other hand, recent molecular studies have shown that two species of *Semenovia* (*S. transiliensis*, *S. dasycarpa*) appear within the *Tetrataenium* Group (Paik & Watson, In press) and two other species (*S. dichotoma*, *S. fragioides*) from a phylogenetic study of Iranian Apiaceae (Yousef *et al.,* In press), appear more closely related to the *H. candicans* (=*Tetrataenium* clade). Both anatomical and molecular study of *Semenovia* support a close relationships with *Tetrataenium*. However, further work on this enigmatic genus is needed, with more extensive sampling beyond the four species, before any taxonomic changes can be proposed.

6.4.6 Conclusion

Fruit anatomy appears to show more useful characters than vegetative characters and those of leaf micromorphology investigated in chapter 5. Allied genera such as *Pastinaca*, *Semenovia*, *Tordylium* and *Zosima* are well supported as distinct from *Heracleum* on the basis of these fruit characters. Fruit anatomy also provides characters to support the separation of the genus *Tetrataenium* from the remainder of *Heracleum*. However, the relationships among these groups remain cryptic. For this reason further investigations were conducted into fruit surface micro-morphology. The results of these studies are presented in the following chapter.

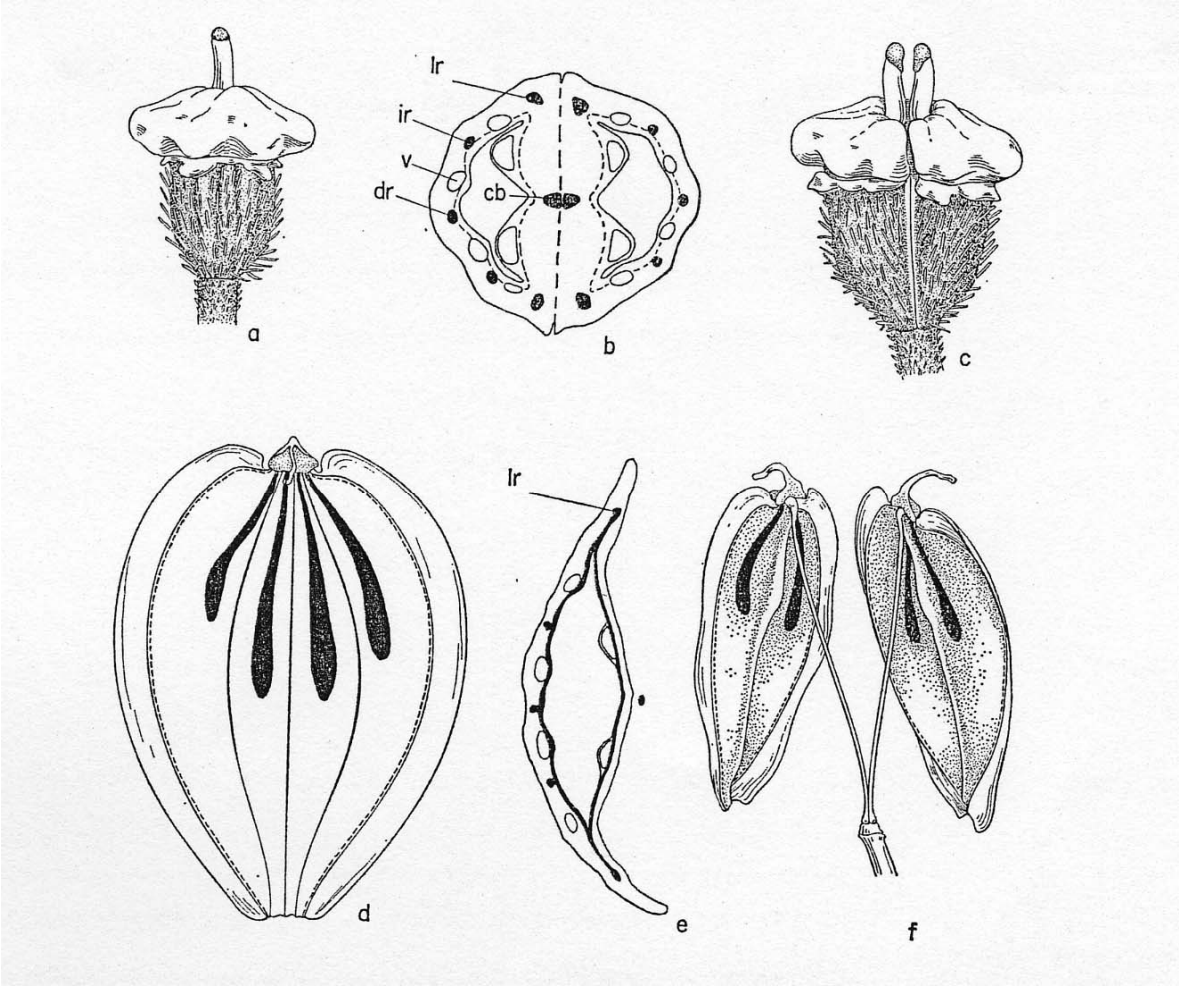


Fig. 6.1 Floral and fruit morphology and anatomy of *Heracleum lanatum* Michx.

(a) Dorsal view of flower shortly after anthesis; (b) median transition (diagrammatic) of developing mericarp shortly after anthesis; (c) lateral view of flower shortly after anthesis; (d) dorsal view of mature fruit; (e) median transaction (diagrammatic) of mature mericarp; (f) lateral view of mature fruit [Details: *cb*, carpophore vascular bundles; *dr*, dorsal rib bundle; *ir*, intermediate rib bundle; *lr*, lateral rib bundle; *v*, vitta (secretory canal)]. Figures from Theobald (1971).

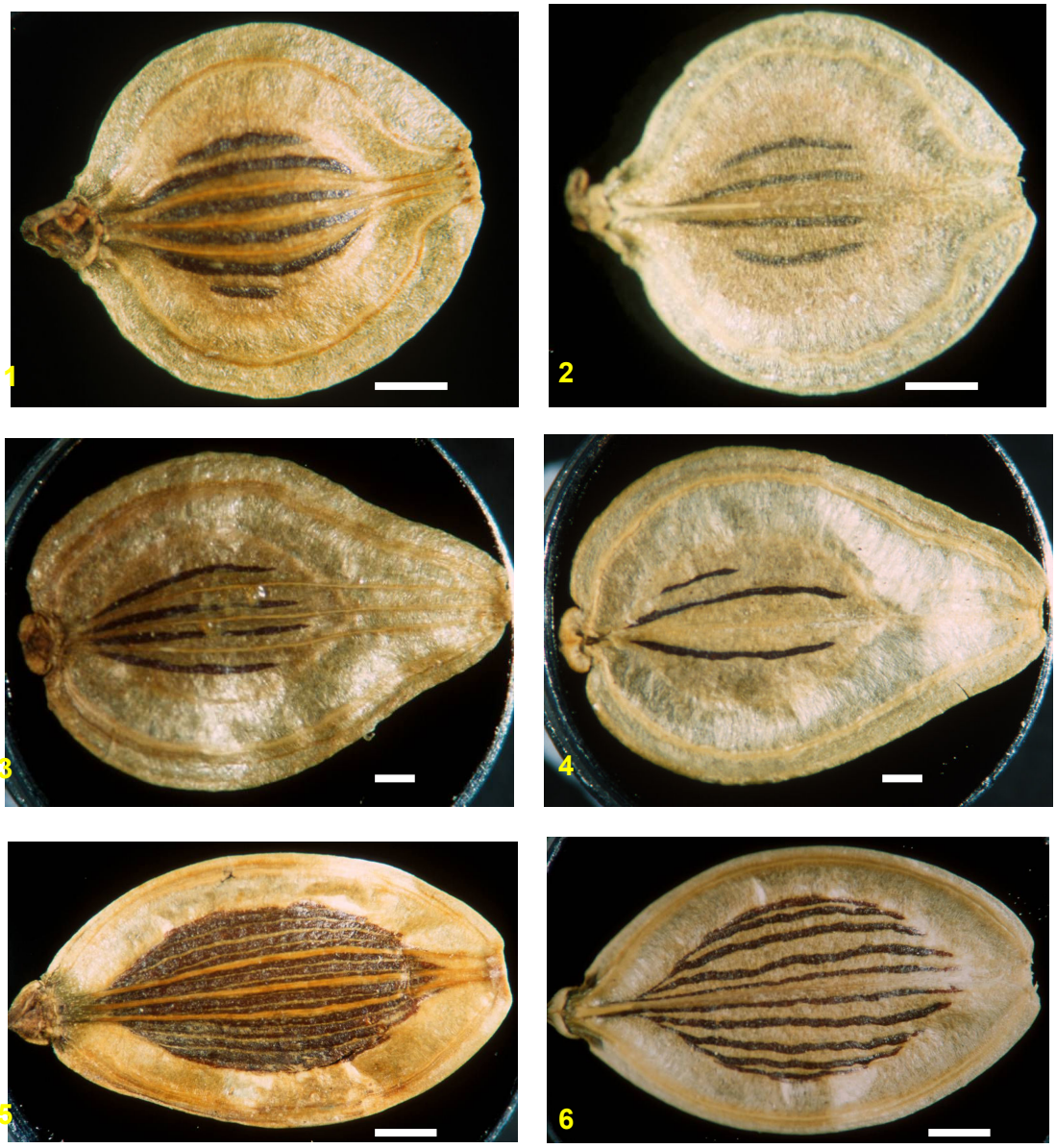


Fig. 6.2 LM photographs of *Heracleum* fruit

1-2. *Heracleum bhutanicum* M.F. Watson (Wood 6811); **3-4**. *H. burmanicum* Kurz. (Hooker 1974); **5-6**. *H. burmanicum* Kurz. (Winet 1800). (1, 3, 5: dorsal surface; 2, 4, 6: commissural surface). Scale bars: 1 mm.

Fig. 6.3 LM photographs of *Heracleum* fruit
1- 2. *Tordyliopsis brunonis* DC. (Hooker *s.n.*); **3 - 4**. *H. candicans* DC. (Yü 14153); **5 - 6**. *H. candicans* DC. (RBGE exp. 264). (1, 3, 5: dorsal surface; 2, 4, 6 commissural surface). Scale bars: 1 mm.

Fig. 6.4 LM photographs of *Heracleum* fruit
1 - 2. *Heracleum candolleanum* (Wight & Arn.) Gamble (White *s.n.*); **3 - 4**. *H. canescens* Lindley (Gamble *s.n.*); **5 - 6**. *H. franchetii* M. Hiroe (Ho *et al.* 1752). (1, 3, 5: dorsal surface; 2, 4, 6: commissural surface). Scale bars: 1 mm.

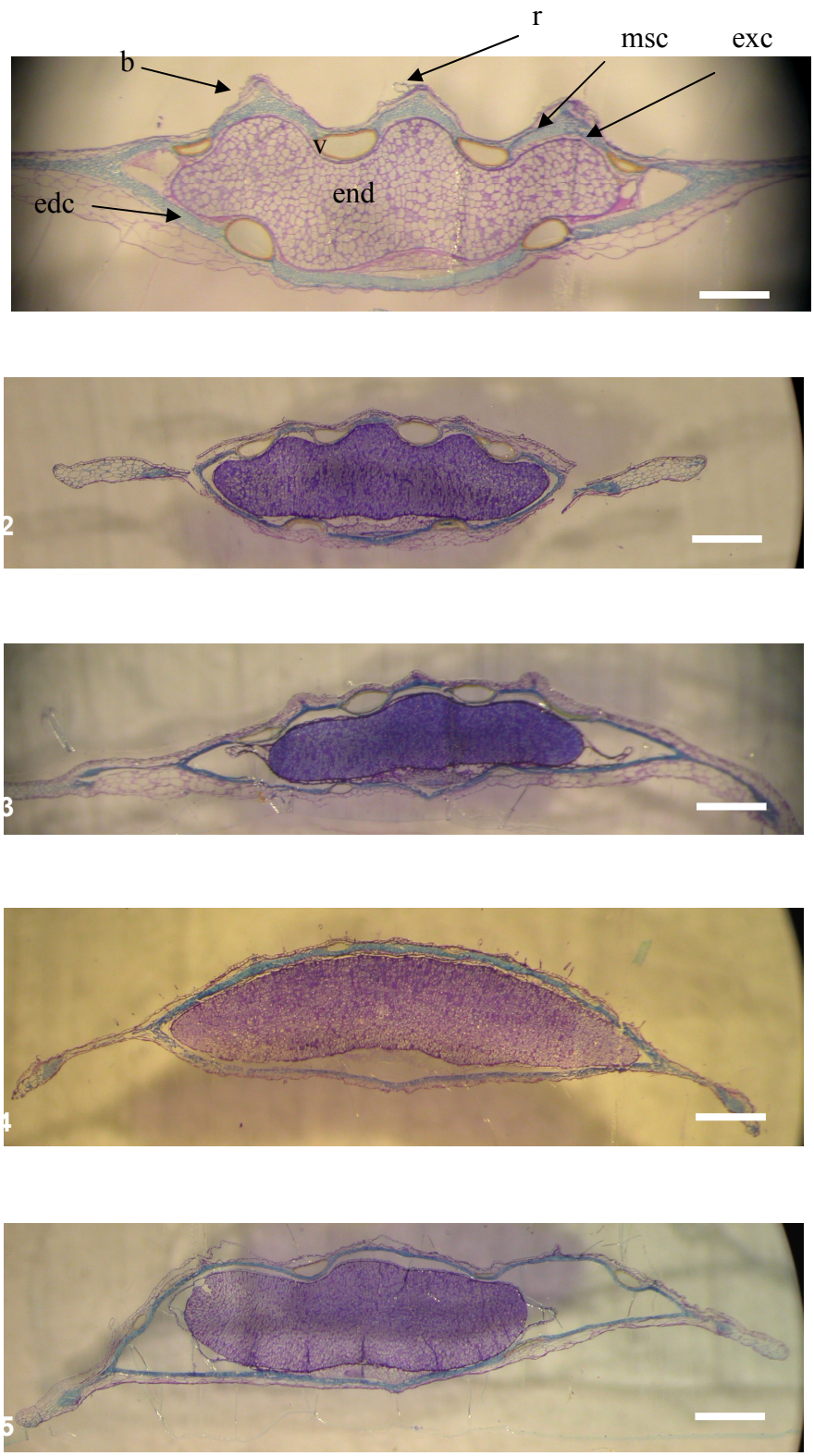


Fig.6.5 Examples of transverse sections of *Heracleum* fruit.

1. *H. nepalense* D. Don (Long & Noltie 21); **2**. *Heracleum candicans* DC. (Yü 14153); **3**. *H. obtusifolium* DC. (Ho *et al.* 2339); **4**. *H. millefolium* Diels (RBGE exp. 78); **5**. *H. kingdonii* H. Wolff (Ace 2137). [end = endosperm; exc = exocarp, msc = mesocarp, edc = endocarp; v = vittae or oil ducts; b = vascular bundle; com = commissural face; r = ridges]. Scale bars: 1 mm.

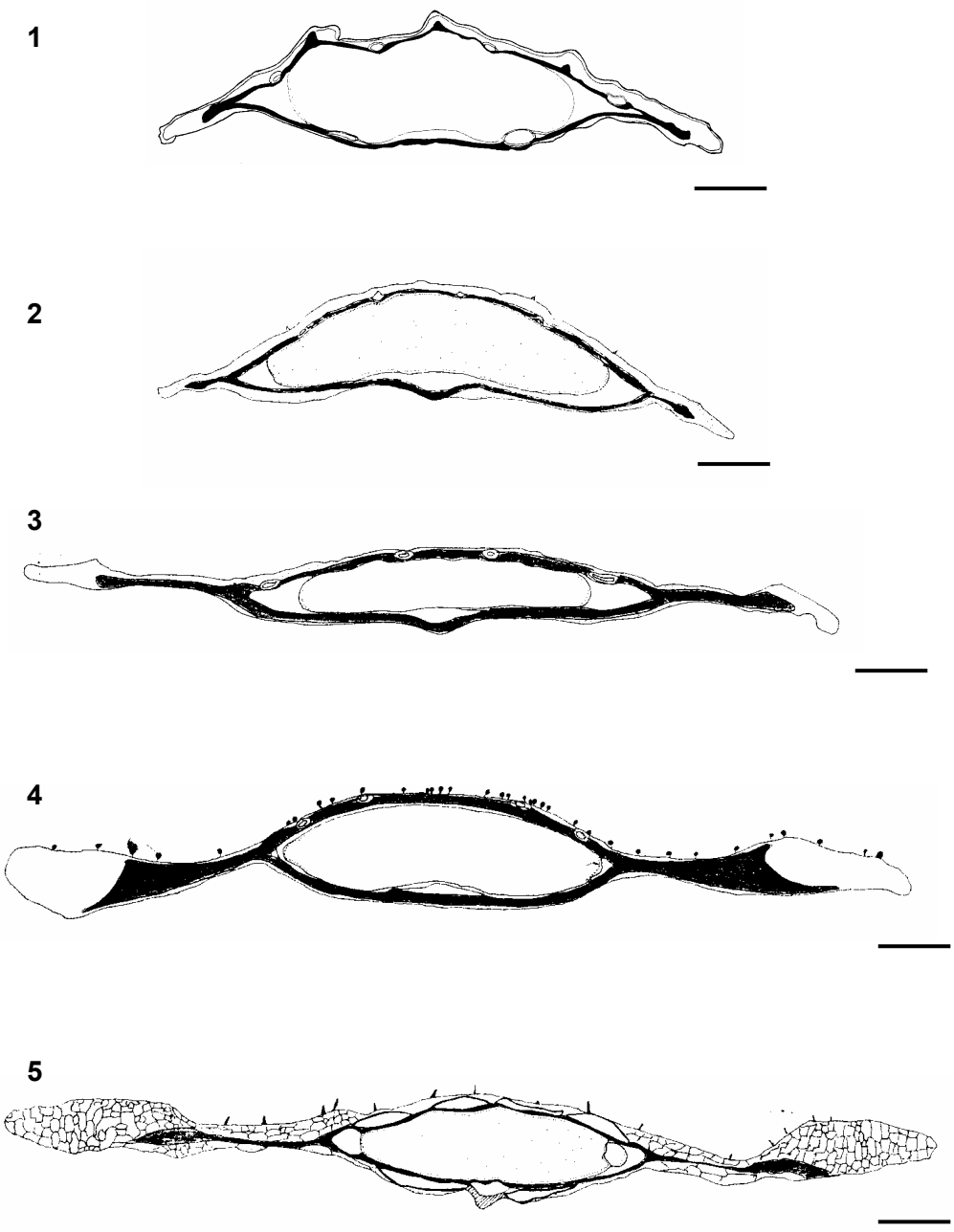


Figure 6.6: Diagram of median transverse sectioning of related species of *Heracleum*.
1: *Pastinaca sativa* L. (Auquier 1694); **2**: *Semenovia transiliensis* Regel & Herd. (Roldugin *s.n.*); **3**: *Tordyliopsis brunonis* DC. (McBeath 2221); **4**: *Tordylium aegyptiacum* Lam. (Lamond & McClintock 2775); **5**: *Zosima absinthifolia* Link (Lamond 3822). Scale bars: 1 mm.

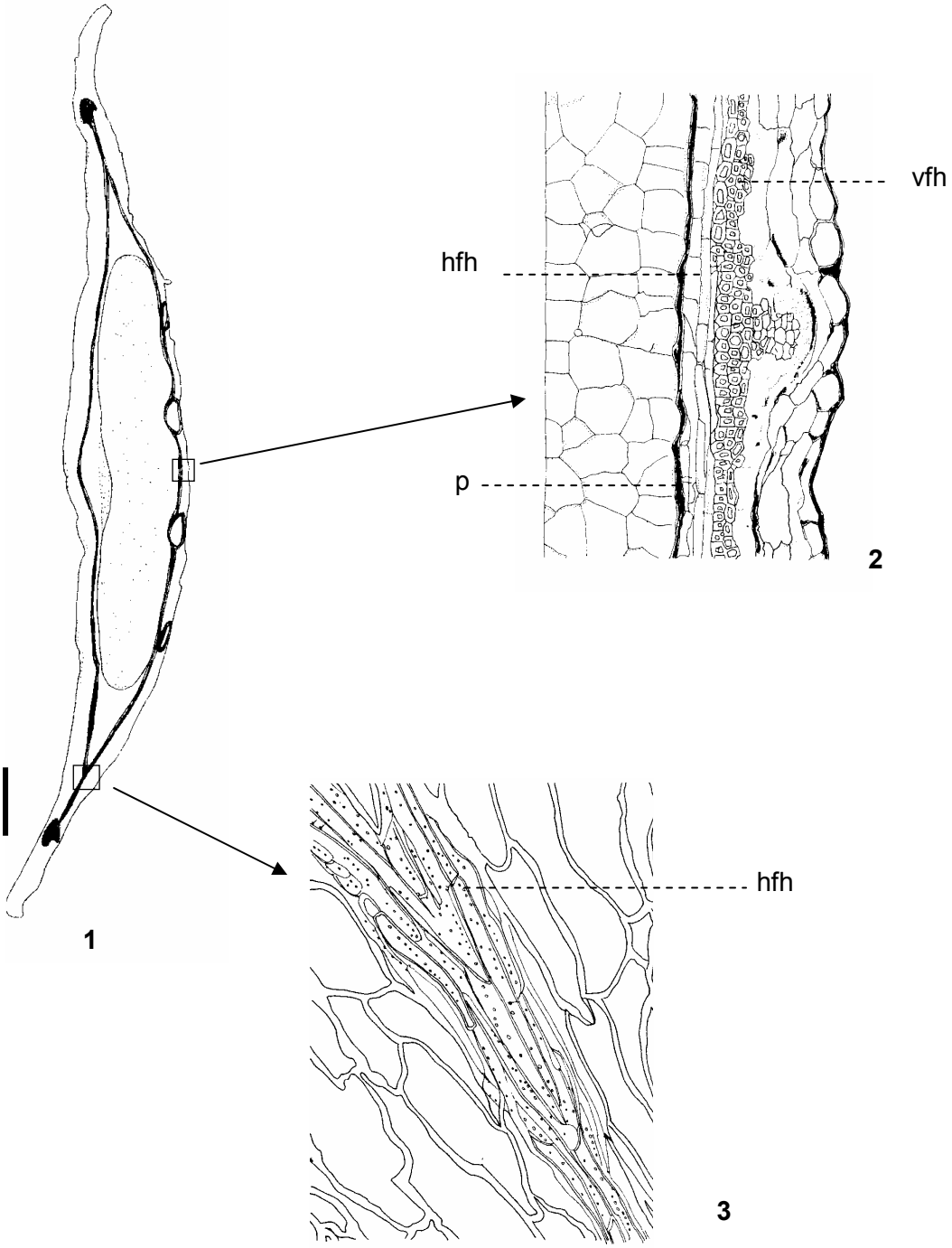


Figure 6.7 Median transverse section of *H. sphondylium* L. (Brumitt 19044).

1: Transverse section of mericarp; **2**: Dorsal ribs and mesocarp; **3**: Seed cavity area (Details: hfh = horizontal fibre-like hypendocarp, vfh= vertical fibre-like hypendocarp, p= parquetry layer). Scale bar= 1 mm.

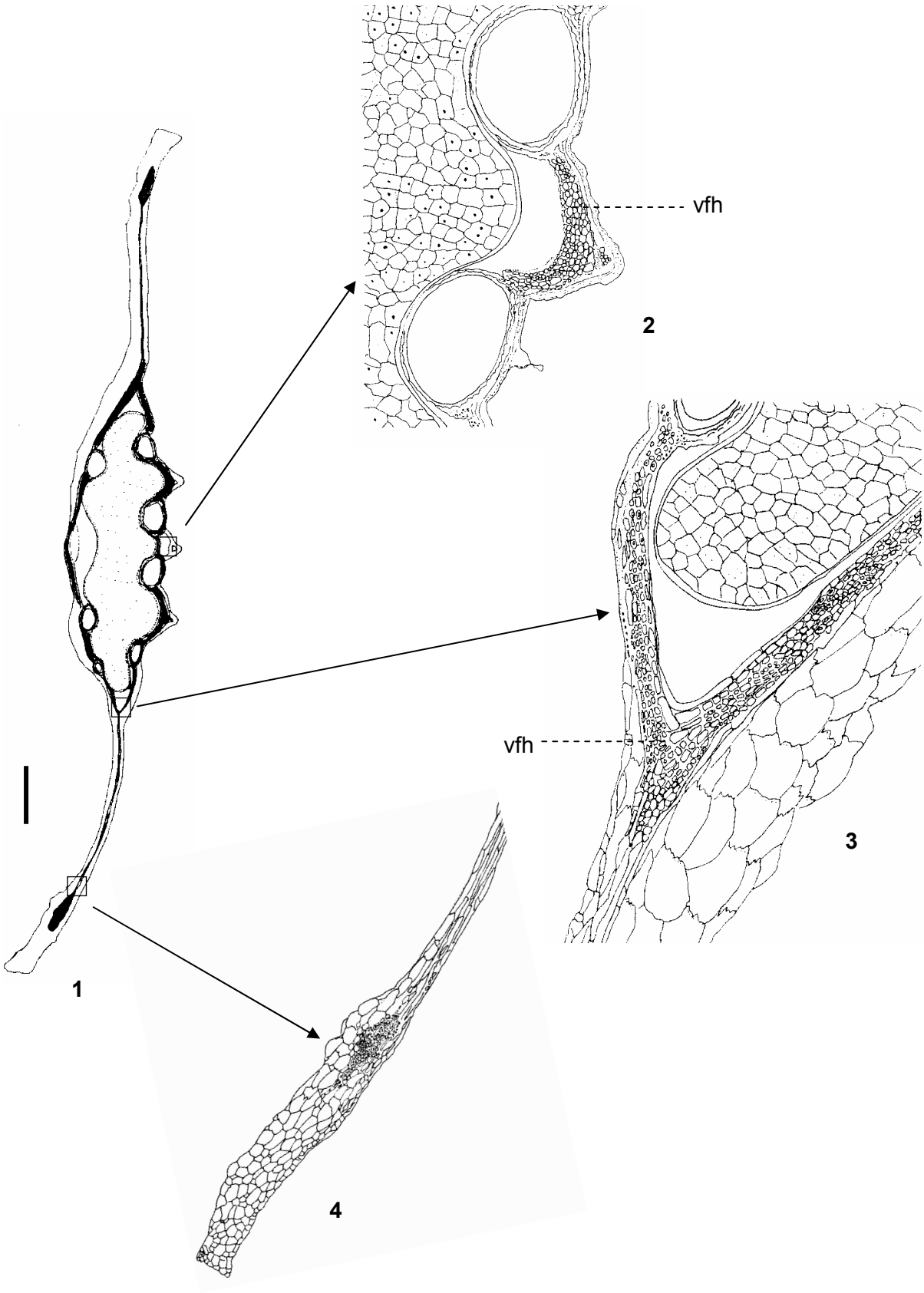


Figure 6.8 Median transverse section of *Heracleum nepalense* D. Don. (Long & Noltie 21). **1**: Transverse section of mericarp; **2**: Dorsal ribs; **3**: Seed cavity area; **4**: Marginal rib (Details: vfh= vertical fibre-like hypendocarp). Scale bar= 1 mm.

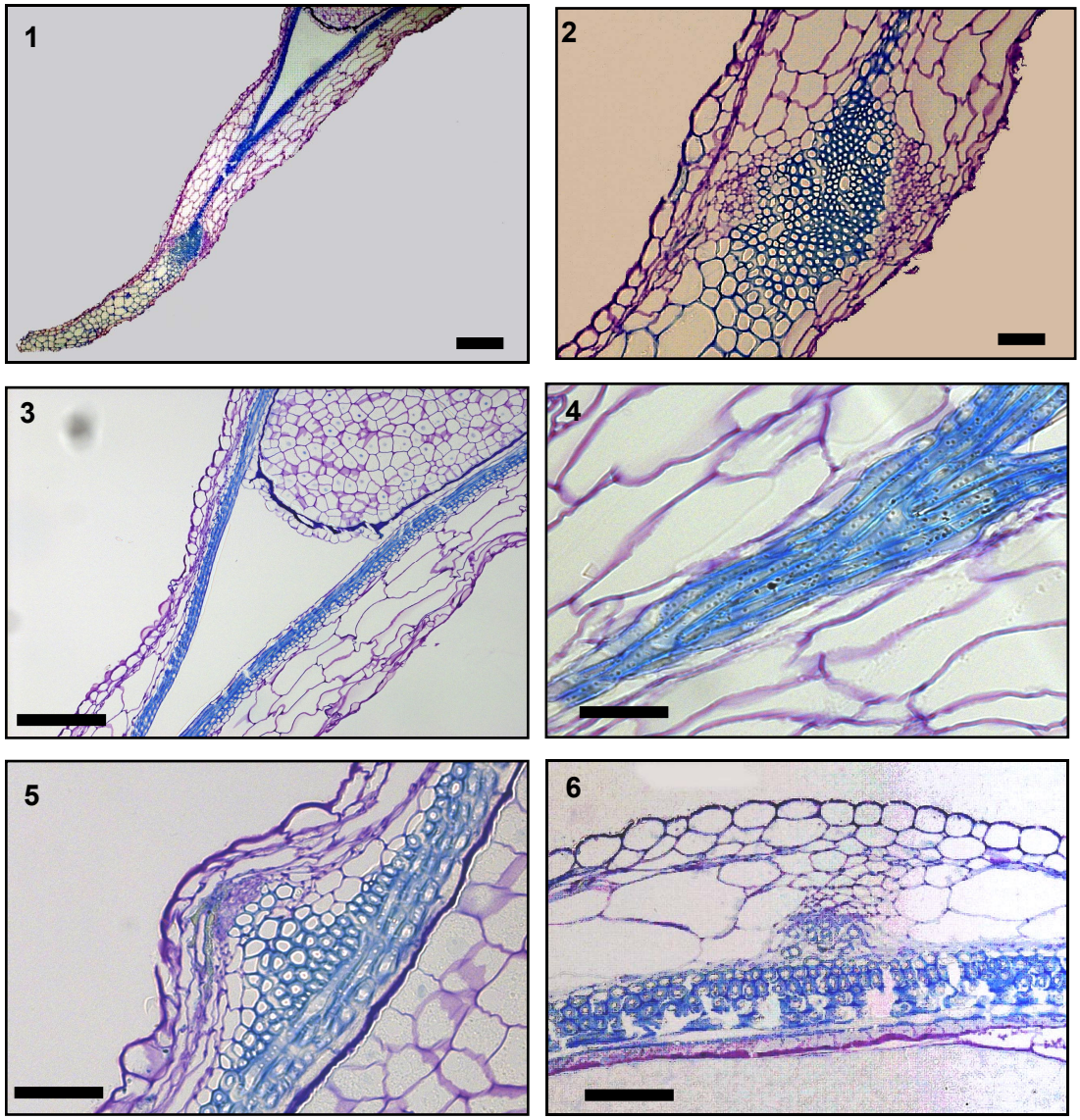


Fig. 6.9 LM micrographs of transverse section of mericarp of *Heracleum sphondylium* L. (Brumitt 19044).
1: Seed cavity and winged marginal rib. **2**: Vascular bundles and a large sclerenchyma massive in the base of inflated part of the marginal rib. **3**: Seed cavity. **4**: Horizontal lignified fibres (hypendocarp) of the seed cavity. **5**: Dorsal rib (Vascular bundles of dorsal ribs are situated in middle of the ribs outer mesocarp, at maturity hypendocarp is thickened under the vascular bundles). **6**: Lateral rib (obsolete, hypendocarp consists of 1 layers of horizontal fibres and 2-3 layers of vertical ones). Scale bars: A = 200 μm; B, D, E = 100 μm; C, F = 50 μm.

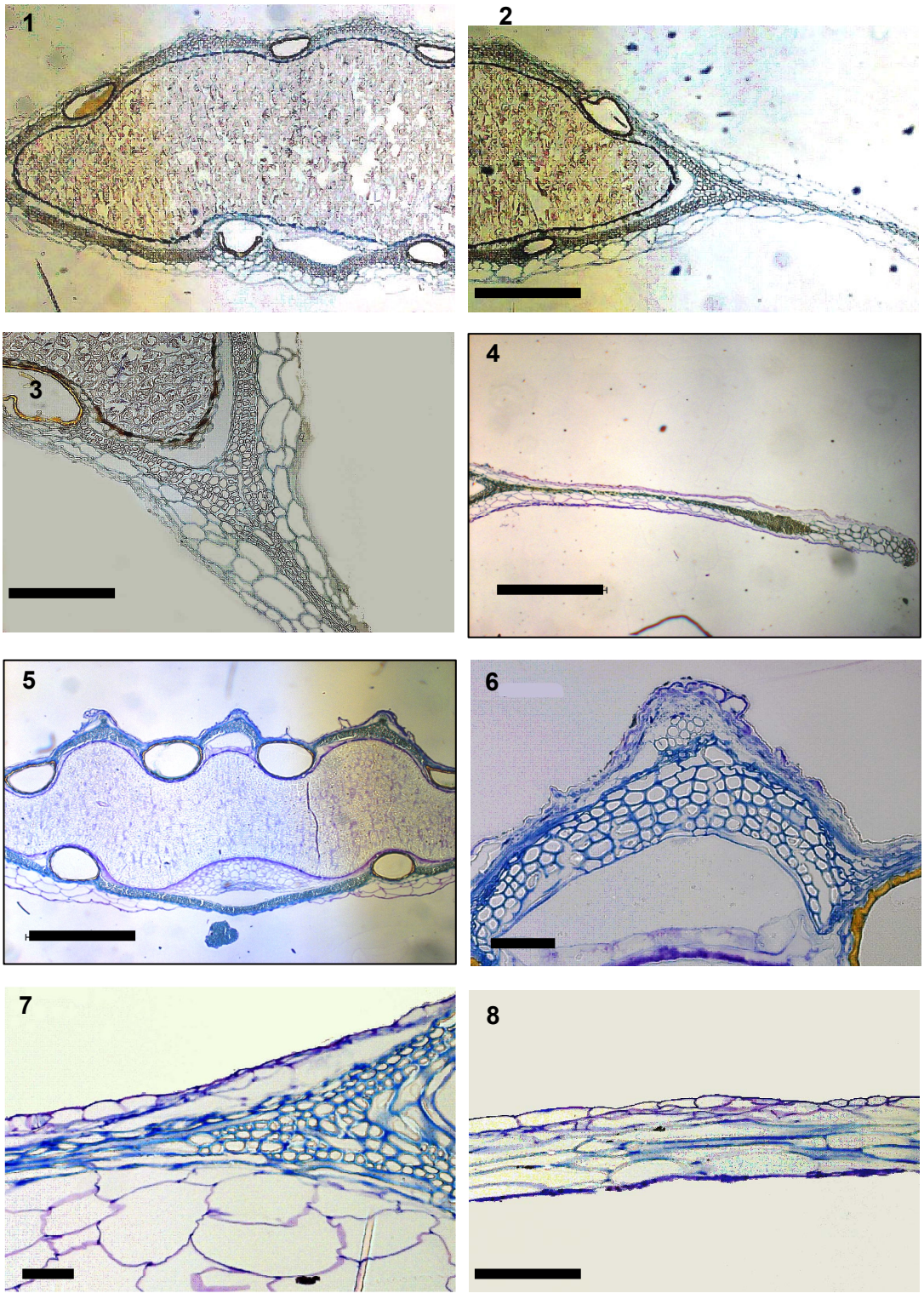


Fig. 6.10 LM micrographs of transverse section of mericarp of *Heracleum bhutanicum* M.F. Watson (1-4: Wood 6811) & *H. nepalense* D.Don (5-8: Long & Noltie 21).
1-2: Fruit body (4 dorsal, & 2 commissural vittae). **3**: Seed cavity and commissural vittae (seed cavity is composed of vfh (=vertical lignified fibres of hypendocarp). **4**: Proximal part & inflated distal part. **5**: Fruit body of *H. nepalense*. **6**: Dorsal ribs (prominent, Vascular bundles in the top of the ribs, hypendocarp well thickened). **7**: Seed cavity (vertical fibres of hypendocarp). **8**: Proximal part of wing (1-2 layers of Horizontal fibres of Hypendocarp). Scale bars: A, B, D, E = 500 μm; C, F = 200 μm; G = 50 μm; H = 100 μm.

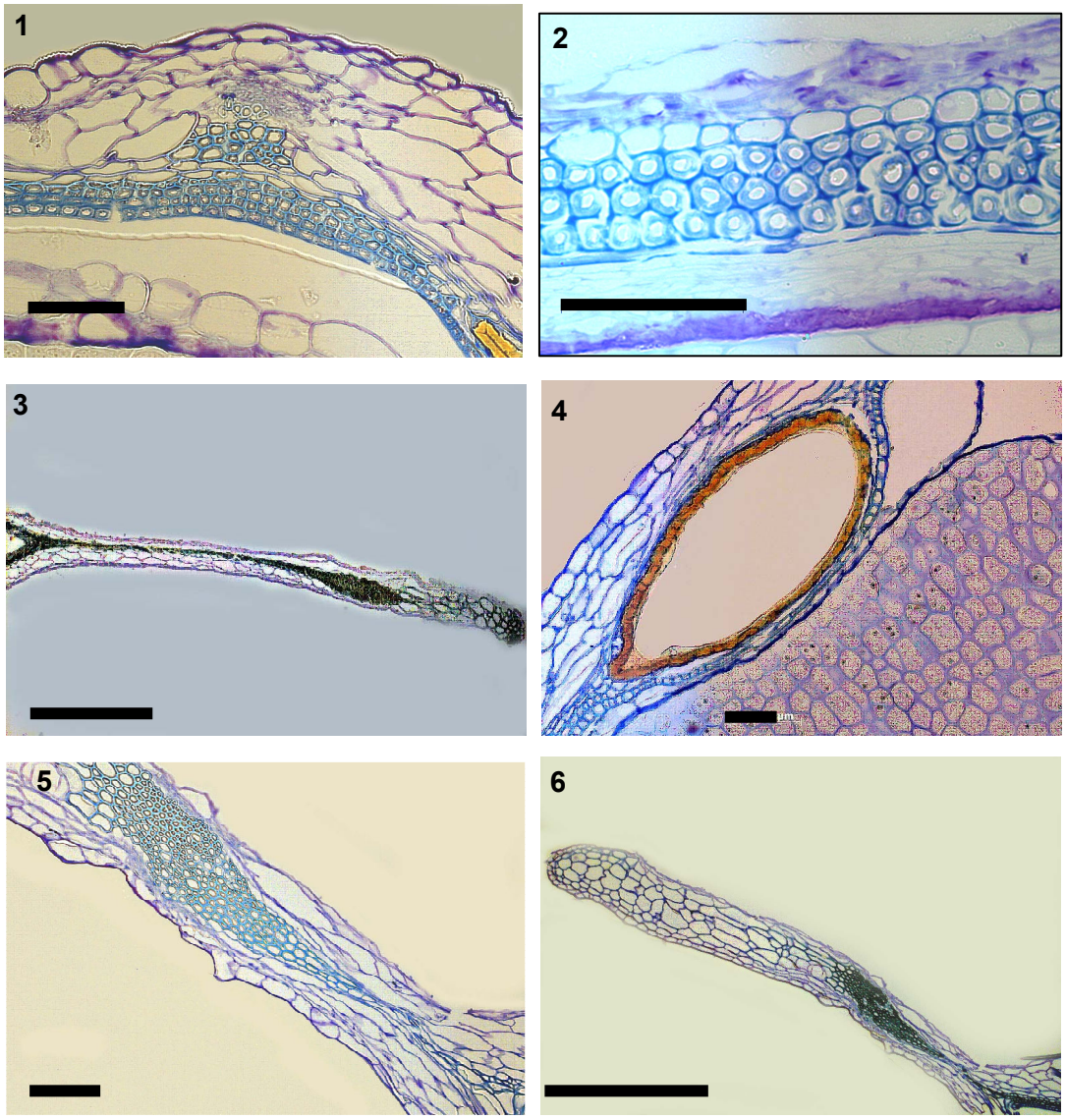


Fig. 6.11 LM micrographs of transverse section of mericarp of *Heracleum obtusifolium* DC. (A-C: Ho *et al*. 2339) *& H. candicans* DC. (D-F: RBGE exp. 264).
1: Dorsal ribs (Vascular bundles in the middle of the ribs, 3-4 vfh) **2**: Intercostal region of dorsal face (3-4 vfh). **3**: Proximal part & inflated distal part. **4**: Dorsal vittae. **5-6**: Short proximal part & well developed inflated distal part. Scale bars: A, B, D = 50 μm; C = 500 μm; E, F = 200 μm.

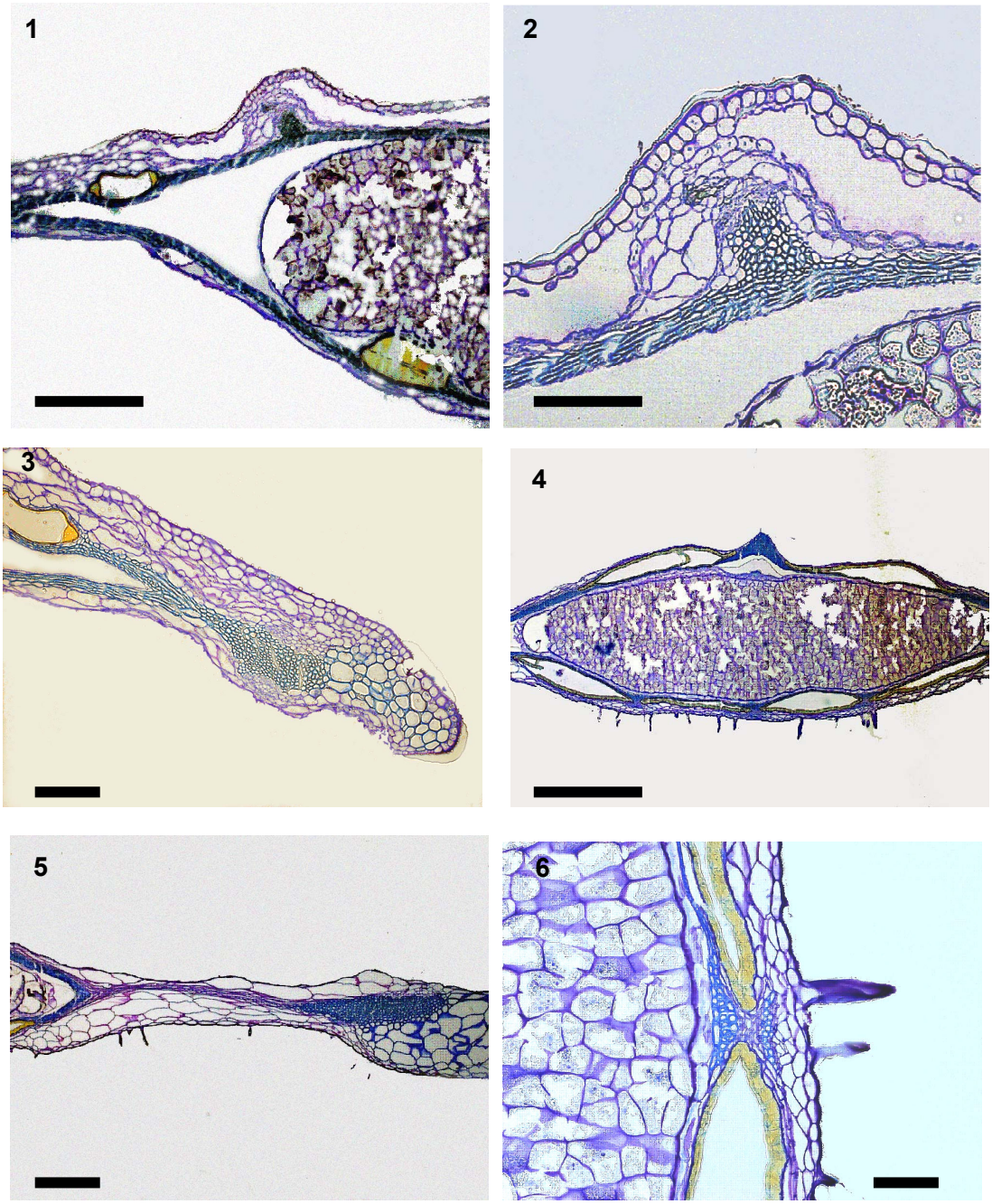


Fig. 6.12 LM micrographs of transverse section of mericarp of *Pastinaca sativa* L. (1-3: Auquier 1694) & *Zosima absinthifolia* Link (4-6: Lamond 3822).
1: Seed cavity. **2**: Dorsal ribs (low-keeled, Vascular bundles in the middle of the ribs hypendocarp that is well thickened). **3**: Proximal part (short, not distinguishable) and Inflated distal part (short). **4**: Fruit body of *Zosima absinthifolia*. **5**: Proximal part. **6**: Dorsal vittae (large, almost occupy the entire areas of the mesocarp between each of the ribs). Scale bars: A, F = 100 μm; B, E = 200 μm; C = 50 μm; D = 500 μm.

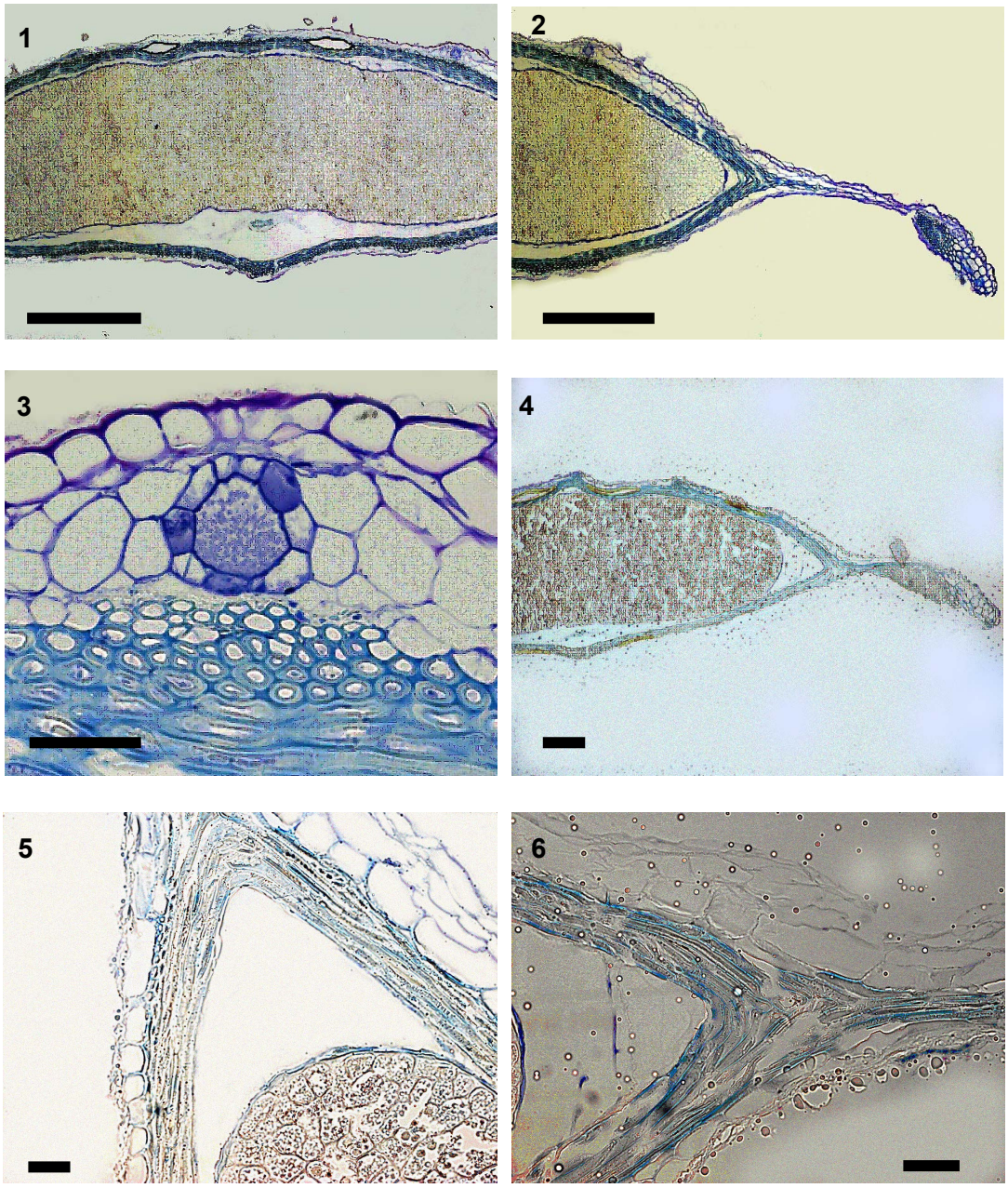


Fig. 6.13 LM micrographs of transverse section of mericarp of *Heracleum millefolium* Diels (1-4: RBGE exp. 78) & *Semenovia transiliensis* Regel & Herd. (5-6: Roldugin *s.n.*). **1**: Fruit body. **2**: Seed cavity and proximal part (seed cavity is composed of hfh (=horizontally fibres of hypendocarp). **3**: Rib secretory ducts. **4**: Fruit body of *Semenovia transiliensis*. **5**: Seed cavity (vertical fibres of hypendocarp). **6**: Proximal part of wing. Scale bars: A, B = 100 μm; C, D, E = 200 μm; F = 50 μm.

Chapter 7: Micro-morpholgy (SEM study of fruit surface)

7.1 Introduction

Heywood & Dakshini (1971) demonstrated the importance of the detailed study of the mericarp to understand better and discriminate between genera of the Apiaceae tribe Caucalideae. Their study was based on the structure of the primary and vallecular (secondary) ridges and the hairs or spines which are found on them. Similarly, in *Heracleum* and allies, the mericarps are valuable in furnishing taxonomic characters. This group of plants has dorsally compressed mericarps with thickened margins and conspicuous or inconspicuous primary ridges. Several studies of taxa allied to *Heracleum* have looked at fruit surface architecture using SEM (Scanning Electron Microscopy). These include *Pastinaca* (Menemen & Jury, 2001a), *Tordylium* (Al-Eisawi & Jury, 1988) and *Zosima* (Menemen & Jury, 2001c). In these studies, the surface between two ribs, the margin of the mericarp surface, hairs present and hair surface characters were found and used at some taxonomic levels to delimit the taxa. Other studies such as Pimenov & Ostroumova's (1994) work on *Malabaila* studied the general fruit morphology and anatomy using light microscopy. These studies have demonstrated the usefulness of fruit surface characteristics in these genera, but to date there has yet to be any published study using SEM on *Heracleum* fruit surfaces. This chapter reports an investigation of the surface architecture of *Heracleum* fruits, in particularly the search for potentially useful taxonomic characteristics of the epidermal surface.

7.2 Material and methods

7.2.1 Materials

A survey of 43 specimens of *Heracleum* and allied genera, including some type specimens was conducted. Material was mainly obtained from dried herbarium specimens, but also one living specimen held at Royal Botanic Garden Edinburgh (*Heracleum* sp. nov.) – see Table 7.1. The entire fruit surface was surveyed for all specimens, and characters recorded for comparision between taxa,

Table 7.1 Fruit materials for SEM study of fruit surface. * not shown in figures.

Species	Source and Voucher
Heracleum austriacum L.	Austria, Salzburg 1400-1500m, M. Eysn *s.n.* (E)
H. barmanicum Kurz	Winet *s.n.* (K)
H. bhutanicum M.F. Watson	Bhutan, Chukka District, Chuka, 1400m, Watson 6811 (E)
H. bivittatum de Boissieu	China, Yunnan, Delavay 6904 (P)
H. cachemiricum Clarke	India, Gamble 24931 (K)
H. candicans DC.	India, Kashmir, Polunin 56/574 (E)
H. candicans DC.	Bhutan, Punakha, Wood 6291 (E)
H. candicans DC.	China, Qinghai, Ho *et al.* 2001 (E)
H. candolleanum (White & Arn.) Gamble	India, Wight 1156 (K)
H. canescens Lindl.	India, Gamble 5710A (K)
H. cashemiricum C.B. Clarke	India, Gamble 24931 (K)
H. dissectum Ledeb.	China, Wang 3579 (PE)
H. fargesii de Boissieu	China, Sichuan, Farges *s.n.* (P)
H. forrestii H. Wolff	China, Yunnan, Forrest 13115 (E)
H. franchetii M. Hiroe	China, Qinghai, Nangqên Xian, Ho *et al.* 1752 (BM)
H. hemsleyanum Diels	China, Hubei, Henry 6469 (K)
H. kingdonii H. Wolff	China, Yunnan, GLGS 8225 (E)
H. lallii Norman	Nepal, Dhevoj 132 (E)
H. lanatum Michx.	USA, Montana, Clements *s.n.* (E)

H. mantegazzianum Sommier & Levier	United Kingdom, two miles from Munlochy, Duncan *s.n.* (E)
H. millefolium Diels	China, Yunnan, RBGE exp. 78 (E)
H. moellendorfii Hance	Korea, Kwangryung, Paik *s.n.* (E)
H. moellendorfii Hance*	Korea, Jumbong Mt., Paik *s.n.* (E)
H. nepalense D. Don	Bhutan, Thimphu, Wood 7146 (E)
H. nepalense D. Don*	Tibet, Cooper 688 (E)
H. nepalense D. Don*	Bhutan, Upper Mo Chu, Sinclair & Long 5342 (E)
H. obtusifolium DC.	China, Qinghai, Ho *et al.* 2339 (E)
H. oreocharis H. Wolff	China, Yunnan, Maire 2558 (E)
H. pinnatum C.B. Clake	India, Hooker, *s.n.* (K)
H. rigens Wall.	India, Wight 1194 (K)
H. scabridum Franch.	China, Yunnan, Delavay *s.n.* (P)
H. sp. (new)	China, Yunnan, Gaoligongshan, Chukai, GLGS 31700 (E)
H. sphondylium L.	United Kingdom, Brummit 19044 (E)
H. stenopterum Diels	China, Yunnan, Wang 17187 (PE)
H. sublineare C.B. Clarke	Nepal, Bheding Dhwoj 272 (E)
H. sublineare C.B. Clarke*	India, Sikkim, Sherabthang, Cooper 814 (E)
H. vicinum de Boissieu	China, Sichuan, Farges *s.n.* (P)
H. wallichii DC.	Nepal, Malemchi, Stainton 6666 (BM)
H. woodii M.F. Watson	Bhutan, Thimphu, Wood 5821 (E)
Malabaila aucheri Boiss.	Turkey, Hakkari, Davis 45280 (E)
Malabaila involucrate Boiss. & Spruner	Greece, Cghanson *s.n.* (E)
Malabaila secacul (Banks & Sol.) Boiss.	Flora of Persia, Azerbaijan, Lamond 3833 (E)
Pastinaca sativa L.	South Africa, Natal, Gordon-Gray *s.n.* (E)
Semenovia dasycarpa Korov	Afganistan, Kapisa, Podlech 12481 (E)
Semenovia transiliensis Regel & Herd.	Kazachstan, Roldugin *s.n.* (NY)
Tordyliopsis brunonis DC.	India, Sikkim, Hooker *s.n.* (K)
Zosima absinthifolia DC.	Flora of Persia, Azerbaijan, Lamond 3822 (E)

7.2.2 Methods

The specimens were first examined under a stereo microscope to assess the maturity of the fruit. Selected samples were then transferred by fine forceps to 12mm carbon discs mounted on 12.5 mm aluminium pin stubs. After placing the stubs in the chamber of a K575x sputter coater (Emitech), the chamber was vacuumed. After 3 min 'High Vacuum Status' was reached on which argon gas was delivered at approximately 2 x 10^{-2} mbar for 20 seconds to evacuate the chamber. Samples were coated with gold palladium at a rate of 25mA for 2.5 minutes. The samples were then studied under a LEO supra 55VP digital scanning electron microscope. Stubs were first scanned at low magnification to select suitable material. The working distance was between 9 and 12 mm and the scanning voltage (EHT) set to 5 kV. Selected images were then saved at several standard magnifications, stored on CD-ROM and also printed out as video prints.

7.3 Results

The following characteristics were seen to potentially offer some taxonomically useful information and were scored for all specimens (Table 7.2)

- surface ornamentation (trichome presence or absence)
- trichome type and surface ornamentation (epicuticular wax striations)
- stylopodium morphology
- calyx teeth presence or absence

Each of these characters has also proven useful in studies of other genera (Al-Eisawi & Jury, 1988; Pimenov & Ostroumova, 1994; Menemen & Jury, 2001a; Menemen & Jury, 2001b).

7.3.1 The surface of mericarp.

The cells on the dorsal surface form a reticulate pattern (figures not shown). The surface between the ribs in all the species is striate, and the margin of the mericarp surface is very similar to that of the surface between the ribs. Trichomes are present on the dorsal surface in all species except *H. barmanicum*, *H. bhutanicum*, *H. candolleanum*, *H. rigens*, *H. sp. nov.* which have a smooth surface (absence of trichomes).

7.3.2 The types of the trichome on the mericarp surface

Three different trichome types are recognised:

(a) Small, triangular-shaped trichomes, which are found in *H. bivittatum*, *H. kingdonii*, *H. nepalense*, *H. woodii*, *Malabaila involucrata*, *Malabaila secacul*, *Pastinaca*

(b) Long, ribbon-shaped trichomes, found in *H. austriacum H. candicans H. cashemiricum, H. dissectum, H. lallii, H. lanatum, H. mantegazzianum, H. moellendorfii, H. millefolium, H. nepalense, H. obtusifolium, H. oreocharis, H. pinnatum, H. shondylium, H. sublineare, H. wallichii, Malabaila aucheri, Semenovia transiliensis, Zosima absinthifolia, Tordyliopsis brunonis*.

(c) Relatively short (compare to ribbon type), acute-headed cylindrical-shaped trichomes, found in *H. fargesii*, *H. forrestii*, *H. franchettii*, *H. hemsleyanum*, *H. oreocharis*, *H. scabridum*, *H. stenopterum*, *H. vicinum.*

7.3.3 Trichome Surface Ornamentation

Distinctive surface ornamentation patterns of trichomes were found in all

investigated species. Three different ornamentation types of hair surface are defined:

(a) Smooth trichome surface, found in *H. bhutanicum*, *H. candicans*, *H. canescens*, *H. cashemiricum*, *H. disectum*, *H. lallii*, *H. lanatum*, *H. mantegazzianum*, *H. moellendorfii*, *H. obtusifolium*, *H. pinnatum*, *H. shondylium*, *H. sublineare*, *H. wallichii*, *Zosima absinthifolia*, *Semenovia transiliensis*, *Semenovia darsycarpa.*

(b) Papillose trichome surface, found in *H. bivittatum*, *H. fargesii*, *H. forrestii*, *H. franchettii*, *H. hemsleyanum*, *H. kingdonii*, *H. millefolium*, *H. nepalense*, *H. oreocharis*, *H. scabridum*, *H. stenopterum*, *H. vicinum*, *H. woodii*, *Malabaila involucrata*, *Malabaila secacul*, *Pastinaca sativa.*

(c) Striate trichome surface, found in *H. millefolium, Zosima absinthifolia*

7.3.4 Stylopodium morphology

Largely, two types of stylopodium shape can be found.

(a) Depressed stylopodium (Fig. 7.9: 3-8; 7.10: 3-4) in *H. wallichii*, *Malabaila aucheri*, *M. involucrate*, *M. secacul*, and *Zosima absinthifolia.* In the second group, all *Malabaila* species share depressed shape of stylopodium.

(b) Low conical or conical-shaped stylopodium in all remaining species

7.3.5 Presence or absence of calyx teeth

The calyx teeth are either prominent or obsolete, the shape is linear to lanceolate or triangular. Mandenova (1982) refered several species of *Heracleum* to

Tetrataenium primarily on the basis of well-developed, persistent calyx teeth. These species also possess other common features such as better developed and narrowly distributed dorsal and lateral ribs, elongate, truly claviform and septate dorsal vittae, but Mandenova used the calyx teeth as the main character to delimit the genra. The species Mandenova included within *Tetrataenium* have a general tendency to be distinct from other *Heracleum* species based on the above characters, however, a careful study of increased number of herbarium specimen study revealed that the calyx-teeth characters are not as reliable as once thought. Development of the calyx teeth has been seen to depend upon fruit position and fertility, as fruits from outer flowers usually have prominent, persistent calyx-teeth but calyx teeth on the inner sterile fruit can be obsolete. Even in *Heracleum sensu stricto*, excluding *Tetrataenium*, in several species the fruit on the outside of the umbels can have prominent persistent calyx-teeth (e.g. *H. fargessii*, *H. vicinum*, *H. henryi*). So this character must be used with care and in combined with other morphological characters.

Table 7.2 Summary of the result of fruit surface study with the Scanning Electron Microscope (SEM)

Taxon	Source and voucher	trichome	trichome type	Trichome surface	Style and stylopodium	Calyx-teeth
Heracleum austriacum L.	Eysn *s.n.* (E)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. barmanicum Kurz	Winet *s.n.* (K)	Absent	-	-	Conical	Prominent
H. bhutanicum M.F. Watson	Watson 6811 (E)	Absent	-	-	Conical	Obsolete
H. bivittatum de Boissieu	Delavay 6904 (P)	Present	Triangular	Papillate	Low Conical	Prominent
H. candicans DC.	Ho *et al.* 2000 (E)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. candicans DC.	Polunin 56/574 (E)	Present	Ribbon	Smooth	Low Conical	Prominent
H. candicans DC.	Wood 6291 (E)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. candolleanum (W. & A) Gamble.	Wight 1186 (K)	Absent	-	-	Low Conical	Prominent
H. canescens Lindely	Gamble 5170A (K)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. cashemiricum C.B. Clarke	Gamble 24931 (K)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. dissectum Ledeb.	Wang 3579 (PE)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. fargesii de Boissieu	Farges *s.n.* (P)	Present	Cylinder	Papillate	Low Conical	Obsolete
H. forrestii H. Wolff	Forrest 13115 (E)	Present	Cylinder	Papillate	Low Conical	Obsolete
H. franchettii M. Hiroe	Ho *et al.* 1752 (E)	Present	Cylinder	Papillate	Low Conical	Obsolete
H. hemsleyanum Diels	Henry 6469 (K)	Present	Cylinder	Papillate	Low Conical	Obsolete
H. kingdonii H. Wolff	GLGS 8225 (E)	Present	Triangular	Papillate	Low Conical	Obsolete
H. lallii Norman	Dhevoj 1929 (E)	Present	Ribbon	Smooth	Conical	Prominent
H. lanatum Michx.	Clements *s.n.* (E)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. mantegazzianum Sommier & Levier	Duncan *s.n.* (E)	Present	Ribbon	Smooth	Conical	Obsolete
H. millefolium Diels	RBGE exp. 78 (E)	Present	Ribbon	Papillate /Striate	Conical	Obsolete
H. moellendorfii Hance	Paik *s.n.* (E)	Present	Ribbon	Smooth	Low Conical	Obsolete

Table 7.2 Summary of the result of fruit surface study with the Scanning Electron Microscope (SEM), Continued

Taxon	Source and voucher	trichome	trichome type	Trichome surface	Style and stylopodium	Calyx-teeth
H. nepalense D. Don	Wood 7146 (E)	Present	Triangular	Papillate	Low Conical	Prominent
H. new spp.	GLGS 31700 (E)	Absent	-	-	Conical	Obsolete
H. obtusifolium DC.	Ho *et al.* 2339 (BM)	Present	Ribbon	Smooth	Low Conical	Prominent
H. oreocharis H. Wolff	Maire 2558 (E)	Present	Cylinder	Papillate	Conical	Obsolete
H. pinnatum C.B. Clarke	Hooker *s.n.* (K)	Present	Ribbon	Smooth	Low Conical	Obsolete
H. rigens DC.	Wight 1194 (K)	Absent	-	-	Conical	Prominent
H. scabridum Franchet	Delavay *s.n.* (P)	Present	Cylinder	Papillate	Conical / Low Conical	Prominent
H. shondylium L.	Brumitt, 19044 (K)	Present	Ribbon	Smooth	Conical / Low Conical	Obsolete
H. stenopterum Diels	Wang 17187 (PE)	Present	Cylinder	Papillate	Low Conical	Obsolete
H. sublineare C.B. Clarke	Lall. Dhwoj 272 (E)	Present	Ribbon	Smooth	Low Conical	Prominent
H. wallichii DC.	Stainton 6666 (BM)	Present	Ribbon	Smooth	Low Conical /Depressed	Prominent
H. woodii M.F. Watson	Wood 5821 (E)	Present	Triangular	Corugate/Papillate	Conical	Prominent
Malabaila aucheri Boiss.	Davis 4528 (E)	Present	Ribbon	Papillate	Depressed	Obsolete
M. involucrate Boiss. & Spruner	Cghanson *s.n.* (E)	Present	Triangular	Papillate	Depressed	Prominent
M. secacul (Mill.) Boiss.	Lamond 3833 (E)	Present	Triangular	Papillate	Depressed	Obsolete
Pastinaca sativa L.	Gordon-Gray *s.n.* (E)	Present	Triangular	Papillate	Low Conical	Obsolete
Semenovia darsycarpa Pimenov & V.N.Tikhom.	Podlech 12481 (E)	Present	Ribbon	Smooth	Conical	Prominent
Semenovia transiliensis Regel & Herd.	Roldugin *s.n.* (E)	Present	Ribbon	Smooth	Conical	Obsolete
Tordyliopsis brunonis DC.	Hooker *s.n.* (K)	Present	Ribbon	Smooth	Low Conical	Obsolete
Zosima absinthifolia DC.	Lamond 3822 (E)	Present	Ribbon	Smooth /Striate	Depressed	Obsolete

7.4 Discussion

From the above evidence it can be shown that *Heracleum* and related genera can be divided into the following groups:

7.4.1 *Heracleum*

Group 1 (Fig. 7.1; 7.2)

H. fargesii, *H. forrestii*, *H. franchettii*, *H. hemsleyanum*, *H. oreocharis*, *H. scabridum* and *H. stenopterum*

This group differs from other species sampled in the genus in having relatively short, cylindrical trichomes, with a pointed apex and papillose surface. This result corresponds well to the previous anatomical study (fruit tranverse sectioning, See Chapter 6: 6.4.3) and molecular result (Fig. 8.1)

7.4.2 Group 2 (Fig. 7.3)

H. austriacum, H. dissectum, H. lanatum, H. mantegazzianum, H. moellendorfii and *H. sphondylium.*

The members of this group include fruit with long ribbon shape trichomes with a smooth surface. The mericarp structure of this group is very similar with *Heracleum* group 1 but the trichome characters are quite different. It is interesting to note that these species come from a wide area (West Eurasia, East Erasia and North America), and yet share a distinctive type of trichome. This group is also well supported by the fruit anatomical study (Chapter 6: 6.4.3).

7.4.3 Group 3 (Fig. 7.4, 7.5, 7.6)

H. candicans, H. canescens, *H. lallii*, *H. obtusifolium*, *H. pinnatum, Semenovia*

transiliensis, S. dasycarpa H. cachemiricum and *H. sublineare.*

This group has relatively short (ribbon type), sharp-headed cylinder-shaped, trichomes with a hollow centre and a smooth surface. This group differs from *Heracleum* group 1 of less densely distributed and smooth surface of the trichomes. Species delimitation of *H. wallichii* and *H. sublineare* has sometimes been confused because of morphological similarity and overlapping geographical distribution. However, based on this study, *H. sublineare* has cylinder-shaped trichomes, but *H. wallichii* does not (trichomes absent). So this new finding seems to be good character for delimitation of species. Two species of *Semenovia* have a similar pattern of trichomes surface with other speices in this group. This grouping is supported by molecular results (See 8.4.4)

7.4.4 Group 4 (Fig. 7.7)

H. bivittatum, *H. kingdonii*, *H. nepalense* and *H. woodii*.

This group has small and triangular-shaped trichomes with a papilliate surface. Dorsal and lateral ridges are closely spaced, and the calyx teeth are well-developed and prominent. Under the dissection microscope these mericarp surfaces look glabrous, but using SEM tiny trichomes are evident and identifiable.

7.4.5 Group 5 (Fig. 7.8)

H. barmanicum, *H. bhutanicum*, *H. candolleanum*, *H. rigens*, and *Heracleum* sp. nov.

This group is circumscribed by the absence of trichomes on the mericarp. These species all originate in SE Asia and S India. This group also shares the characters of an extra 1 or 2 vittae on the dorsal and commissural sides and a waxy epidermal surface.

7.4.6 *Pastinaca* and *Malabaila* group (Fig. 7.9)

These genera all share mericarp features of widely spaced, obsolete dorsal and lateral ridges, calyx teeth absent or obsolete (as in *Heracleum* group 1), and sparsely distributed, small triangular-shaped trichomes with a papillose surface. Previous mericarp transverse sectioning supports this grouping (Chapter 6: 6.4.4). It is worth noting that *Malabaila aucheri* appears rather different to others in this group: it has relatively long, flat, sharp-headed ribbon-like trichomes. This requires further study, but may be interpreted as an extreme form of the triangular trichomes.

7.4.7 Other taxa (Fig. 7.10)

Heracleum millefolium, *Tordyliopsis brunonis* and *Zosima absinthifolia* have unusual characters that mark them apart from the groups outlined above. These species share long, ribbon-shaped trichomes which are distributed all over the mericarp. Between the species each has distinct characters:

- *Heracleum millefolium* has trichomes with a papillate surface, whereas the others are smooth.
- The trichomes of *Tordyliopsis brunonis* are sparsely pubescent all over the mericarp but densely pubescent mainly on the stylopodium basal area only.
- In *Zosima absinthifolia* the stylopodium is depressed, whereas in *Heracleum millefolium* and *Tordyliopsis brunonis* it is conical.

A research group from Sichuan investigated fruit macromorphology (He *et al*., 1998), and combined these data with petiole and pollen characters in a total 35 characters

from which they produced cladograms for the relationships among W. Chinese *Heracleum* (Zhao *et al*., 2004). Their fruit macromorphological results broadly agrees with the results of the micromorphological analyses presented here. Their Sect. *Heracleum* corresponds to Groups 1 and 2 of the above results. They also accepted Sect. *Villosa* Manden. which corresponds well with Group 3 in this study. Their new section, *Plurivittata* Pu & X.J. He contained *H*. *wenchuanense* (not sampled here), *H*. *bivittatum* (Group 4) and *H*. *barmanicum* (Group 5). This may indicate that these two trichome types may have a close relationship. Unfortunately, differences in taxon sampling make more direct comparisons difficult.

7.5 Conclusion

Although this study of the mericarp of *Heracleum* and related genera is far from complete it is clearly shows that an examination of the micro-morphological characters of mericarp using SEM, particularly trichome type and surface pattern of trichomes, is very useful to delimit generic groups. It can also give some potentially useful characters to indicate relationships within the component genera. The diversity of mericarp surface structure found here is surprisingly varied and much further work will have to be undertaken to consider variability and adaptive significances of the features of surface ornamentation. In the following chapter (chapter 8) I compare the distribution of these morphological character types with a molecular phylogeny of *Heracleum* and allies in order to determine if these characters do reflect a phylogenetic structure.

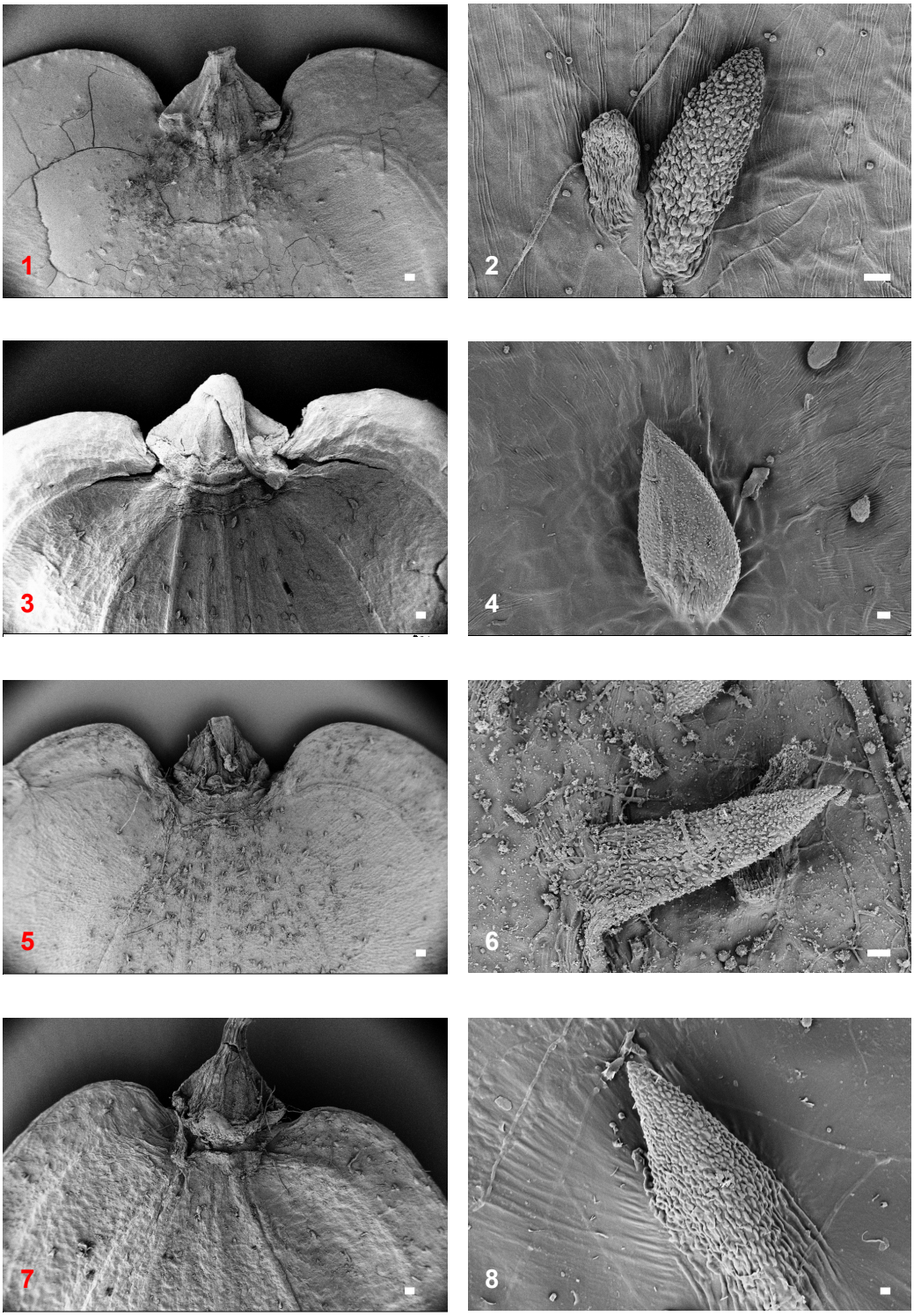


Fig. 7.1 SEM micrographs of the mericarp surface of *Heracleum*.
1-2: *H. hemsleyanum* Diels (Henry 6469, K); **3-4**: *H. forrestii* H. Wolff (Forrest 13115, E); **5-6**: *H. fargesii* de Boissieu (Farges *s.n.*, E); **7-8**: *H. scabridum* Franch. (Delavay *s.n.*, P). Scale bars: 1, 3, 5, 7 = 100㎛; 2, 4, 6 = 10㎛; 8 = 2㎛.

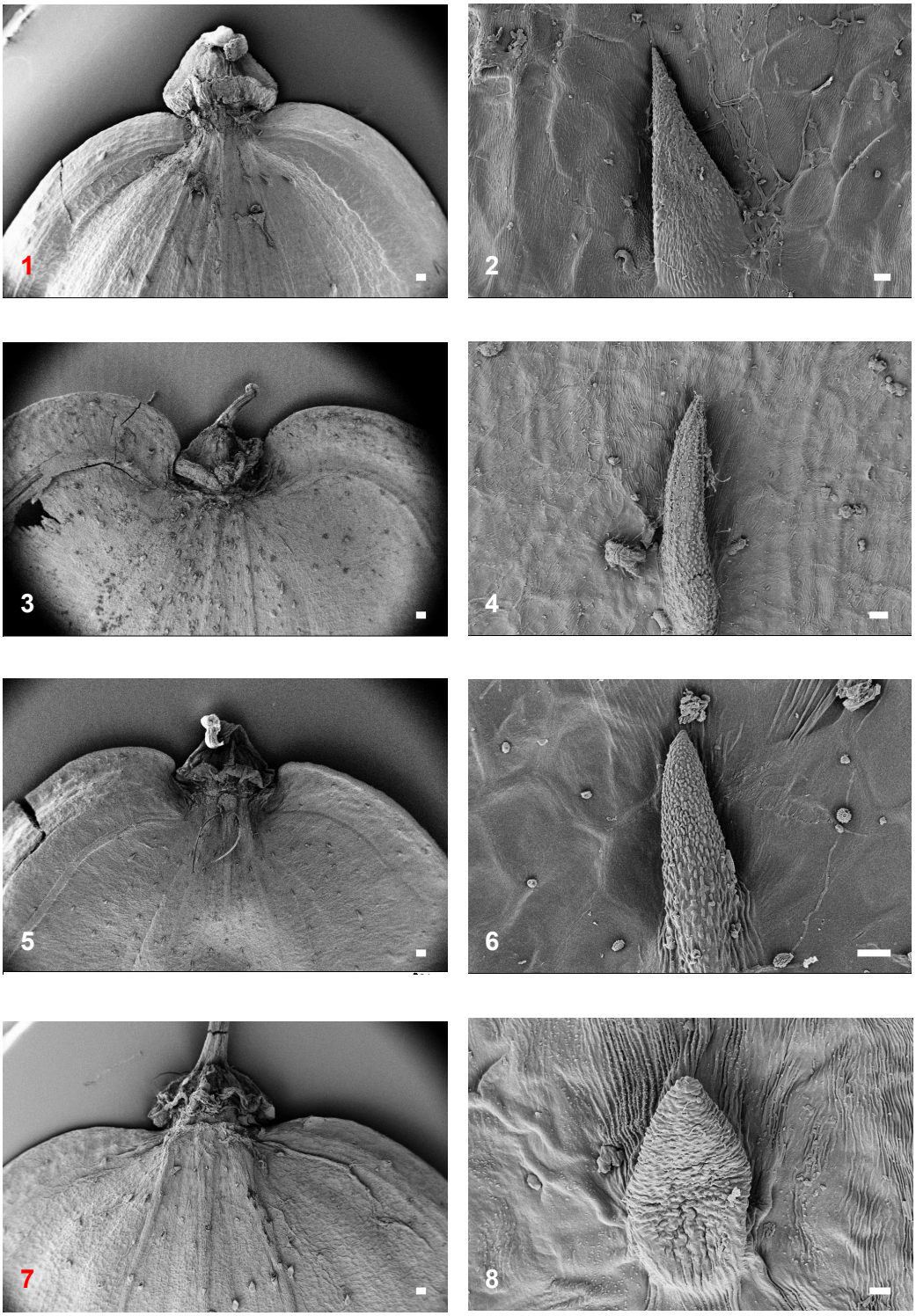


Fig. 7.2 SEM micrographs of the mericarp surface of *Heracleum*.

1-2: *H. oreocharis* H. Wolff (Maire 2558, E); **3-4**: *H. vicinum* de Boissieu (Farges *s.n.*, P); **5-6**: *H. stenopterum* Diels (Wang 17187, PE); **7-8**: *H. franchetii* Hiroe (Ho *et al.*1752, E). Scale bars: 1, 3, 5, 7 = 100㎛; 2, 4, 6, 8 = 10㎛.

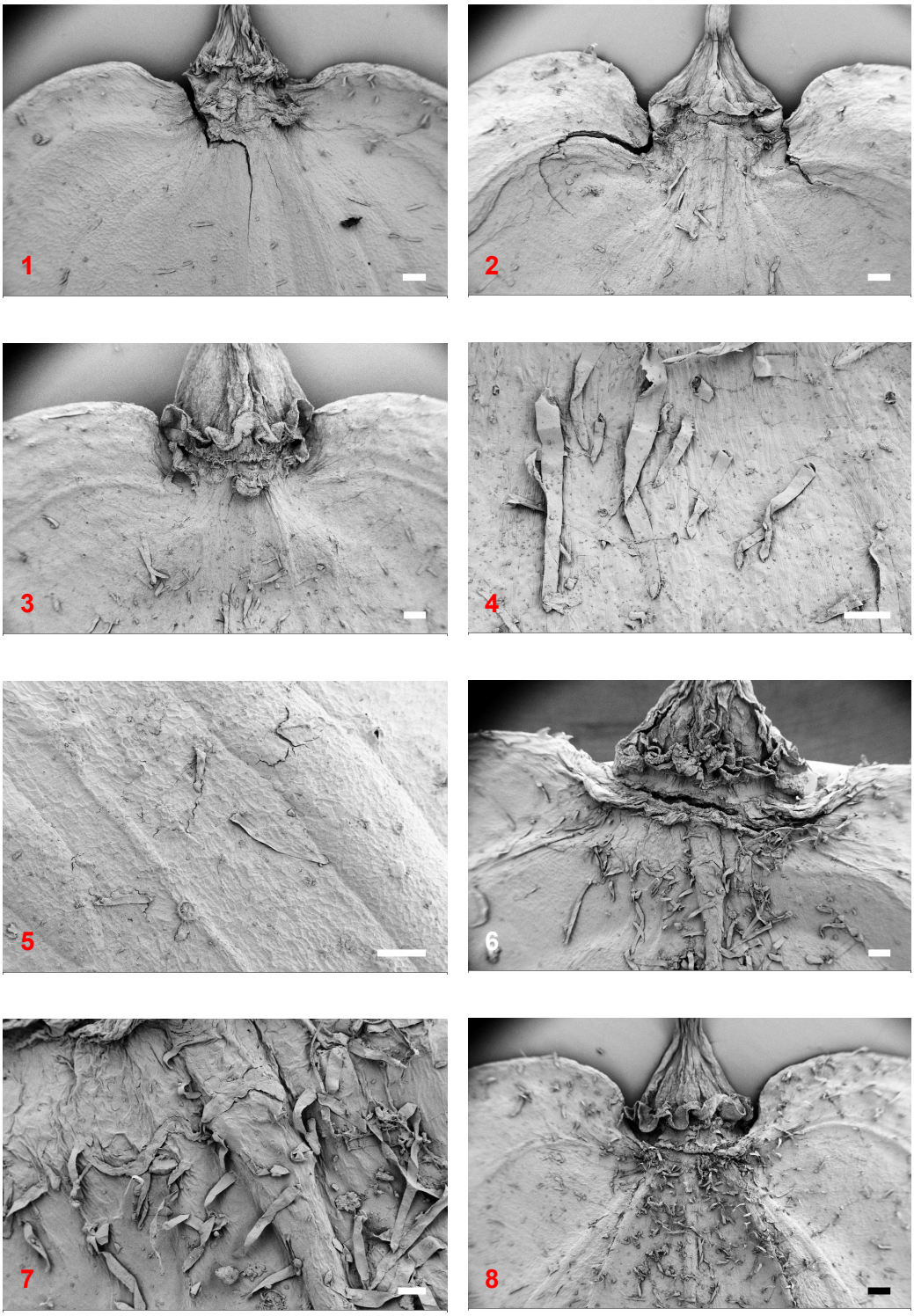


Fig. 7.3 SEM micrographs of the mericarp surface of *Heracleum*.

1: *H. austriacum* L. (Eysn *s.n.*, E); **2**: *H. sphondylium* L. (Brummit 19044, K); **3-4**: *H. dissectum* Ledeb. (Wang 3579, PE); **5**: *H. moellendorfii* Hance (Paik *s.n.*, E); **6-7**: *H. mategazzianum* Sommier & Levier (Duncan *s.n.*, E) **8**: *H. lanatum* Michx. (Clements *s.n.*, E). Scale bars: 1, 2, 3, 5, 6, 8 = 200㎛; 4, 7 = 100㎛.

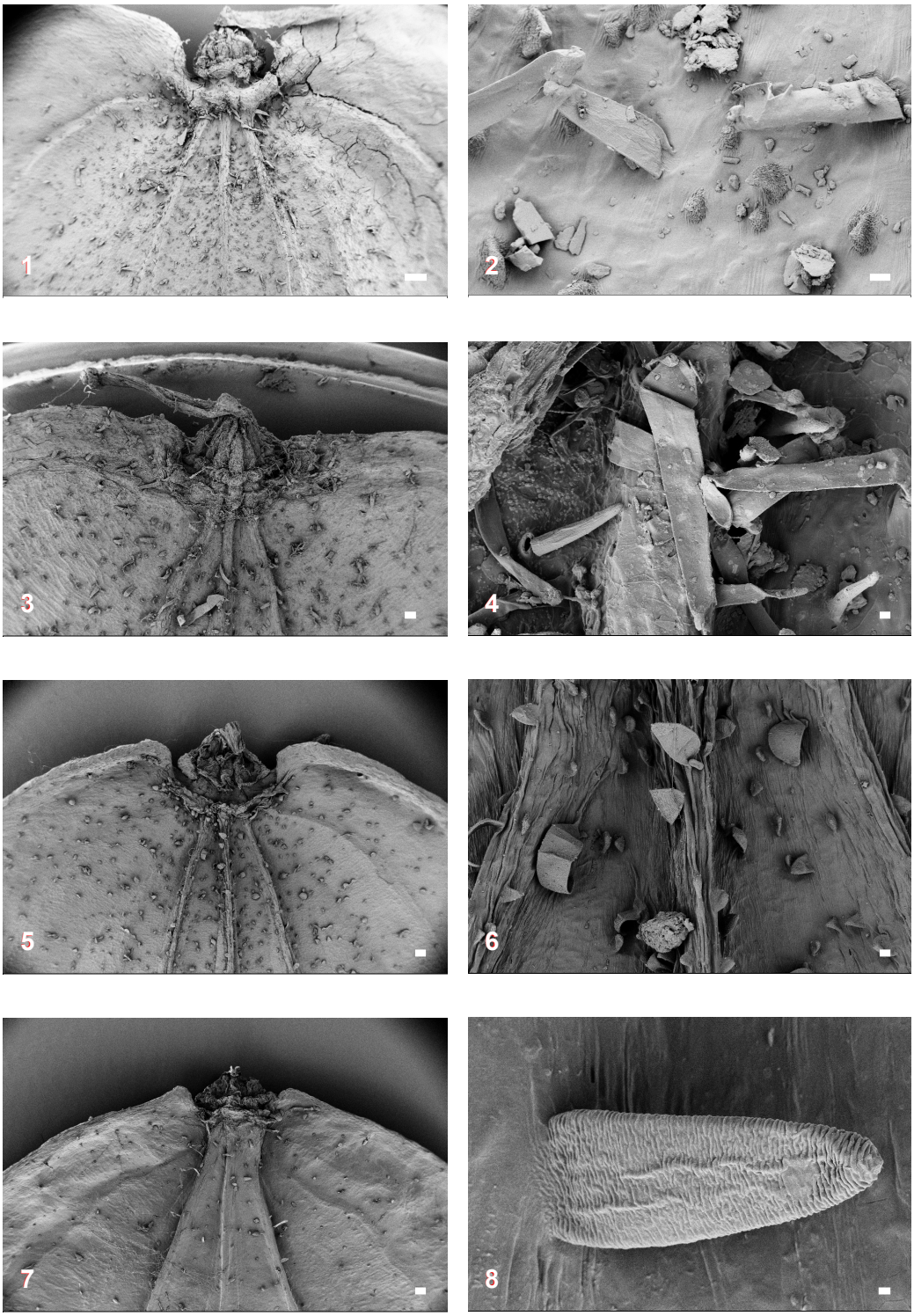


Fig. 7.4 SEM micrographs of the mericarp surface of *Heracleum*.

1-2: *H. pinnatum* C. B. Clake (Hooker *s.n.*, K); **3-4**: *H. canescens* Lindl. (Gamble 5710A, K); **5-6**: *H. candicans* DC. (Polunin 56/574, E); **7-8**: *H. candicans* DC. (Wood 6291, E). Scale bars: 1 = 200㎛; 2, 4 = 10㎛; 3, 5, 7 = 100㎛; 6 = 20㎛; 8 = 3㎛.

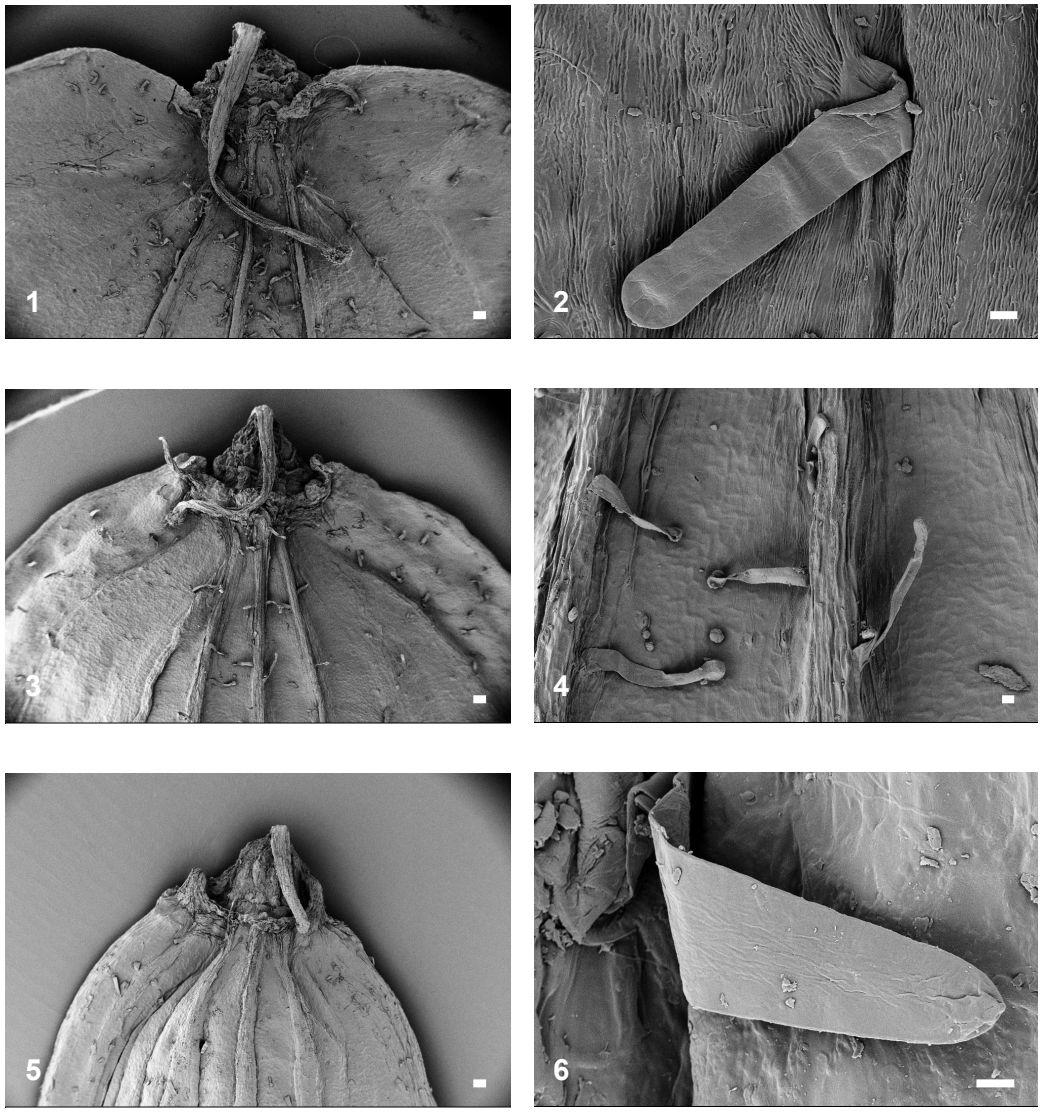


Fig. 7.5 SEM micrographs of the mericarp surface of *Heracleum*.
1-2: *H. obtusifolium* DC. (Ho *et al.* 2339, E); **3-4**: *H. candicans* DC. (Ho *et al.* 2001, E); **5-6**: *H. lallii* Norman (Dhevoj 132, E). Scale bars: 1, 3, 5 = 100㎛; 2, 6 = 10㎛; 4 = 20㎛.

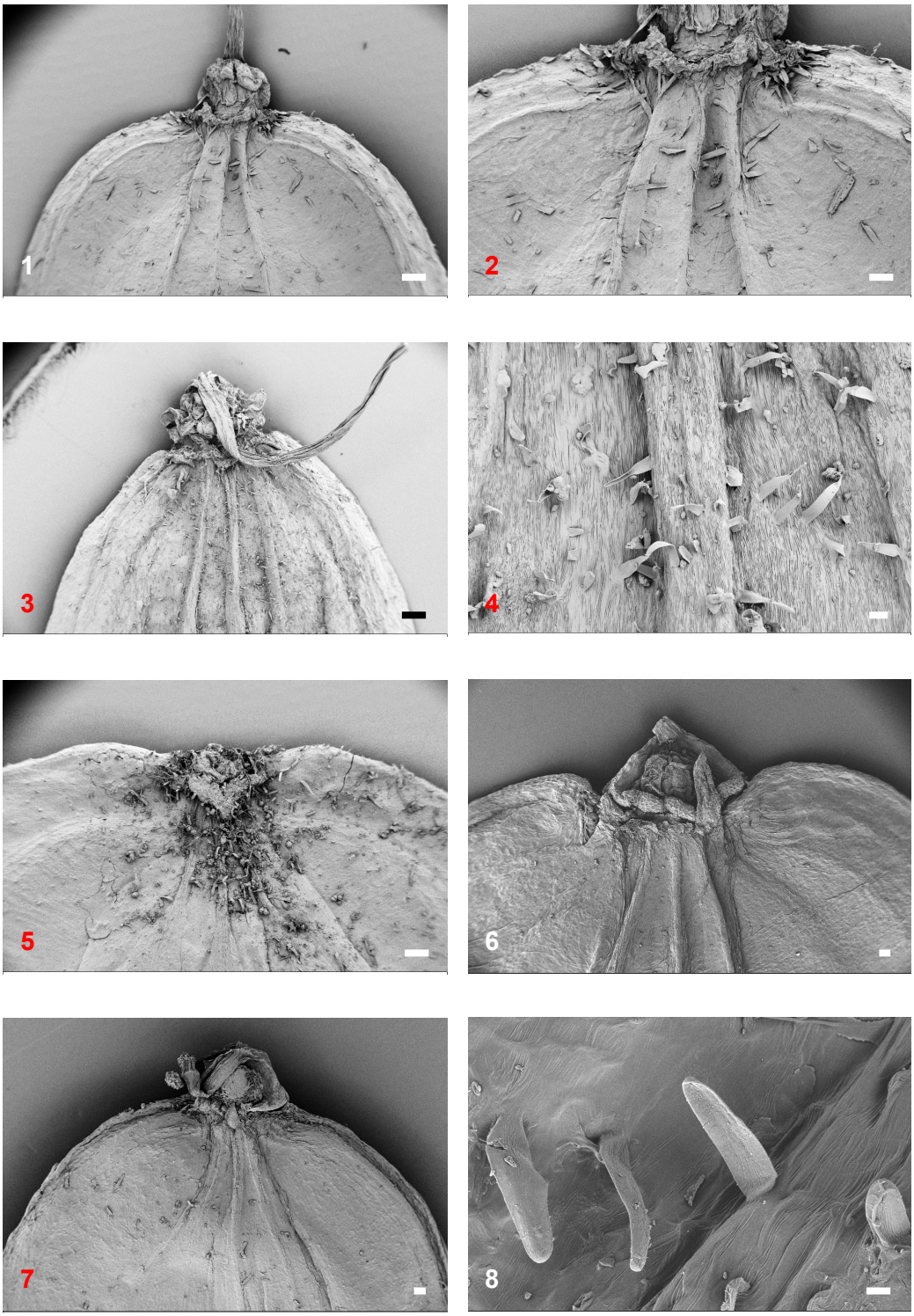


Fig. 7.6 SEM micrographs of the mericarp surface of *Semenovia* & *Heracleum*.

1-2: *S. transiliensis* Regel & Herd. (Roldugin *s.n.*, NY); **3-4**: *S. dasycarpa* Korov (Podlech 12481, E); **5**: *H. cachemiricum* C. B. Clarke (Gamble 24931, K); **6**: *H. wallichii* DC. (Stainton 6666, BM); **7-8**: *H. sublinear* C.B.Clarke (Dhwoj 272, E). Scale bars: 1, 3, 5 = 200㎛; 2, 6, 7 = 100㎛; 4 = 20㎛; 8 = 10㎛.

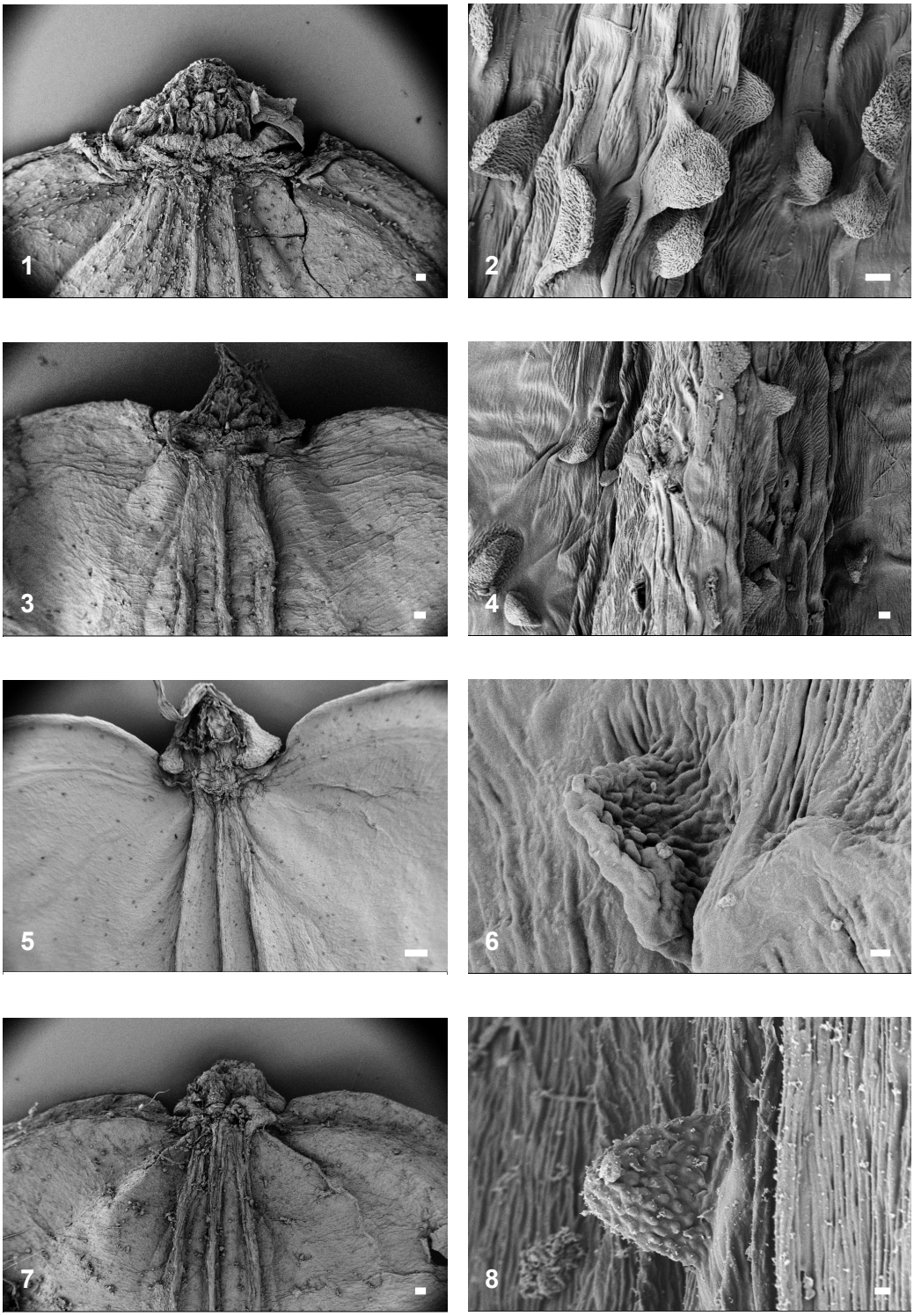


Fig. 7.7 SEM micrographs of the mericarp surface of *Heracleum*.
1-2: *H. woodii* M. Watson (Wood 5821, E); **3-4**: *H. nepalense* D. Don (Wood 7146, E); **5-6**: *H. kingdonii* H. Wolff (GLGS 8225, E); **7-8**: *H. bivittatum* de Boissieu (Delavay 6904, P). Scale bars: 1, 3, 7 = 100㎛; 2, 4 = 10㎛; 6 = 2㎛; 8 = 3㎛.

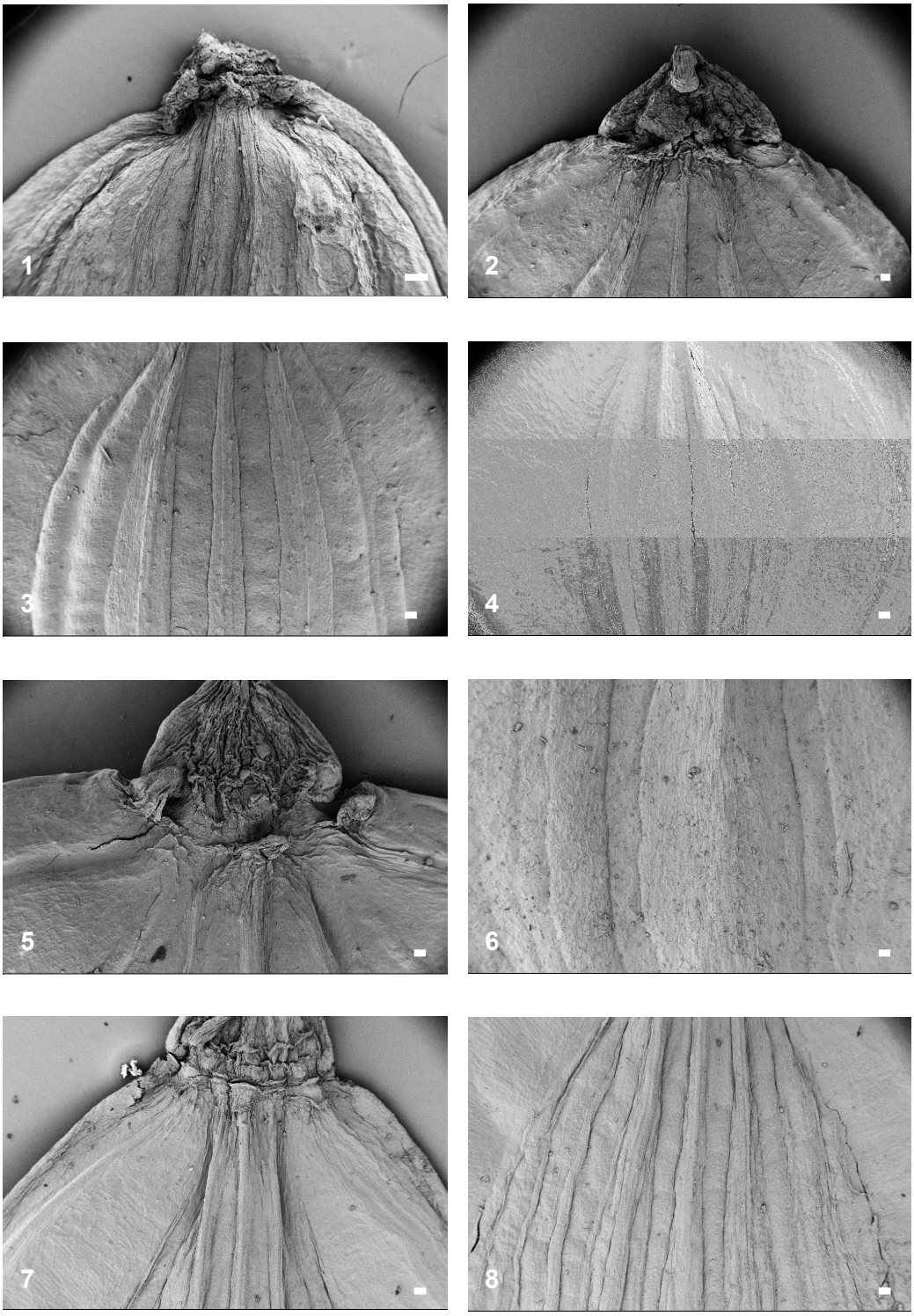


Fig. 7.8 SEM micrographs of the mericarp surface of *Heracleum*.

1: *H. new spp.* (GLGS 31700, E); **2-3**: *H. rigens* Wall. (Wight 1194, K); **4**: *H. bhutanicum* (Watson 6811, E); **5-6**: *H. candolleanum* (Wight 1156, K); **7-8**: *H. barmanicum* (Winet *s.n.*, K). Scale bars: 1 = 200㎛; 2, 3, 4, 5, 6, 7, 8 = 100㎛.

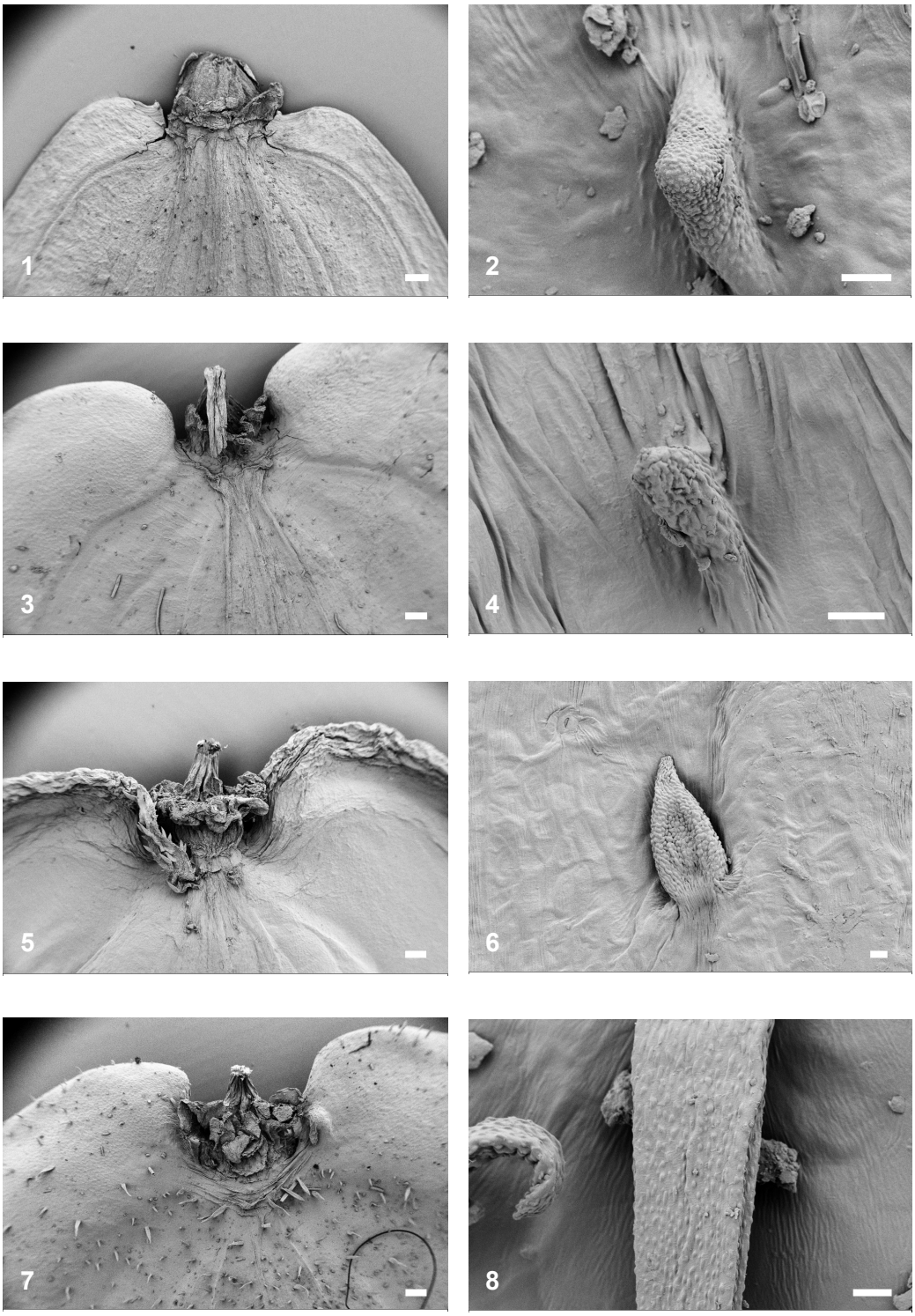


Fig. 7.9 SEM micrographs of the mericarp surface of *Pastinaca* & *Malabaila*.
1-2: *P. Sativa* L. (Gordon-Gray *s.n.*, E); **3-4**: *M. secacule* (Banks & Sol.) Boiss. (Lamond 3833, E); **5-6**: *M. involucrata* Boiss. & Spruner (Cghanson *s.n.*, E); **7-8**: *M. aucheri* Boiss. (Davis 45280, E). Scale bars: 1, 3, 5, 7 = 200㎛; 2, 4, 6, 8 = 10㎛.

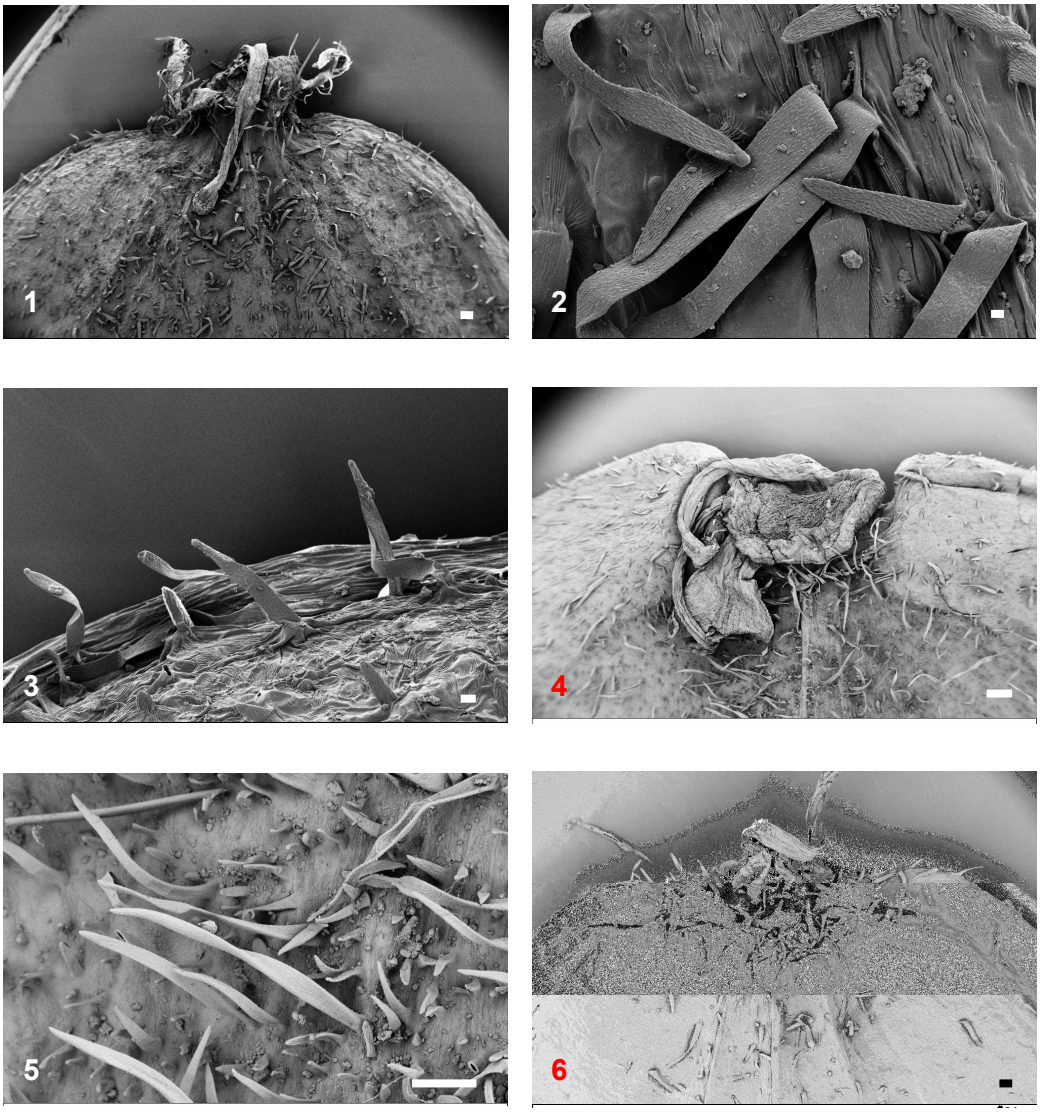


Fig. 7.10 SEM micrographs of the mericarp surface of *Heracleum millefolium, Zosima absinthifolia,* and *Tordyliopsis brunonis.*
1-2: *H. millefolium* Diels (RBGE exp. 78, E); **3-4**: Z. *absinthifolia* DC. (Lamond 3822, E); **5-6**: *Tordyliopsis brunonis* C.B.Clarke (Hooker *s.n.*, K). Scale bars: 1, 5, 6 = 100㎛; 2 = 10㎛; 3 = 20㎛; 4 = 200㎛.

Chapter 8: Phylogenetic Reconstruction and Infrageneric Classification

8.1 Introduction

High level molecular phylogenies have provisionally identified a '*Heracleum* clade' (or 'Major *Heracleum* clade'; in this chapter I use these two terms as same meaning) within the Apioid superclade comprising: *Tetrataenium*, *Tordylium*, *Heracleum* and *Pastinaca*, and possibly *Conium*, *Malabaila* and *Zosima* (Katz-Downie *et al*., 1999; Downie *et al*., 2000a & b; Downie *et al*., 2001). Among the genera sampled within their '*Heracleum* clade', to date only *Heracleum* has representatives in the Sino-Himalayan region (Nepal eastwards to SW China), and even then, sampling within *Heracleum* has so far been limited. However, the Sino-Himalayan region is very important to the understanding of *Heracleum* and its related genera as there are ten *Heracleum* species in the Himalaya and about 30 species in China, and the morphological diversity seen in this region is rather atypical of the genus as a whole (see Chapter 3). Furthermore, the East Himalaya/SW China region is one of two centres of diversity of this widespread genus, the Caucasus region being the other. Problems of morphological complexity and blurred generic boundaries in this region highlight further highlight the importance of more comprehensive sampling from this area in molecular phylogenies. Very recently, Logacheva *et al*. (In press) studied the Western Asiatic taxa within the *Heracleum* clade and suggested that *Heracleum* forms a polyphyletic clade which has poor concordance with previous classifications.

De Candolle (1830) produced the first infrageneric classification which included Himalayan *Heracleum*. In his treatment of *Heracleum* he divided the 26 species known at that time into six sections based on the number of commissural vittae of the fruit. He included four Himalayan *Heracleum* species, placing them in two new sections: *Tetrataenium*: *H. nepalensis*, *H. obtusifolium*, *H. regense* and *Trichogonium*: *H. wallichii* (see Chapter 2 for further discussion). Section *Trichogonium* was not used in later revisions, but Section *Tetrataenium* was elevated to generic rank by Mandenova (1959, 1982) to include these and other species mainly from the Central Himalayan region and South India.

In China there had been no comprehensive taxonomic treatment of *Heracleum* until the publication of *Flora Reipublicae Popularis Sinicae* (Shan & Sheh, 1992). He *et al*. (1998) attempted to the first infrageneric classification of the Chinese species, and recognized five Sections based on fruit morphology along with combined data from petiole anatomy, pollen morphology and cytology (see Table 2.1). This work generally agreed with Mandenova's system and established a new section *Plurivittata* to include the unusual SW Chinese taxa which have one or two additional vittae between the dorsal ribs and on the commisural face of the fruit. Hedge & Lamond (1992) have also commented on the unusual characteristics of the *Heracleum* species in this regions, suggesting that they blur the generic limits with *Pucedanum* and *Angelica* include list of genera. Later, a research group from Sichuan (Zhao *et al*., 2004) undertook a cladistic study to demonstrate the relationships among W. Chinese *Heracleum*. They used general fruit morphology with the combined characters of pollen and petiole anatomy scored for almost same voucher specimens used by He *et al*. (1998). This study

suggested that Western Chinese *Heracleum* should be divided into four or possibly five Sections: Sect. *Millefolia*, Sect. *Plurivittata*, Sect. *Villosa*, Sect. *Heracleum*, and possibly Sect. *Wendia* (*H. apaense*). However, the samples used in this study were few and the identification of some species was ambiguous, and so the new classification (He *et al*., 1998; Zhao *et al*., 2004) was not adopted in the *Flora of China* (Pu & Watson, 2005) which is simply a modified and revised version of *Flora Reipublicae Popularis Sinicae* (Shan & Sheh, 1992).

This chapter is aimed at extending previous molecular studies to include representatives of *Heracleum* and related genera from the Sino-Himalayan region to assess these past taxonomic treatments and establish a robust phylogeny.

1. Our first objective is to evaluate the monophyly of *Heracleum*, determine the validity and monophyly of segregate genera, and determine species relationships.
2. The second objective is to evaluate relationships between related allies of *Heracleum* included in the '*Heracleum* clade' as a basis for future research.
3. The third objective is to evaluate the previously suggested infrageneric classifications of *Heracleum* as they relate to the Sino-Himalaya region.

Results from the ITS and rps16 analyses have been submitted as manuscript to *Taxon* (Paik & Watson, submitted; Appendix 3).

8.2 Materials and Methods

8.2.1 Molecular tools

The suitability of the nuclear ITS region and the rps16 chloroplast region to resolve inter- and intra- generic phylogenies has already been successfully shown in a range of studies on Apiaceae (Downie and Katz-Downie, 1996, 1999; Downie *et al*., 1998, 2000a ; Lee and Downie 1999, 2000) as well as in angiosperms in general (Baldwin *et al*., 1995; Oxelman *et al*., 1997). In these studies both regions have resolved phylogenetic relationships down to species level, and both these regions were used in the present study. For a subset of the taxa, we also examined variation from the cpDNA psbA-trnH spacer locus to see how useful this would be. This region has been successively tested in other plant families (Kress *et al*., 2005) to reveal phylogentically useful information at around the generic and subgeneric levels, but has not been used much before in *Heracleum*.

8.2.2 Taxon sampling

On the basis of several previous studies (Katz-Downie *et al.,* 1999; Downie *et al*., 2000a & b; Downie *et al*., 2001) *Conium maculatum*, *Apium graveolens*, *Ammi majus*, *Anethum graveolens*, and three *Angelica* species were used as outgroups. These outgroups are strongly supported as being distinct from the '*Heracleum* Clade' and so highly unlikely to be part of the in group in the present study. Complete ITS1 and ITS2 sequences for 50 taxa are reported here for the first time (Appendix 1). These are combined with 14 previously published ITS sequences (Downie & Katz-Downie, 1996) in an ITS data matrix of 64 taxa. Data from the intervening 5.8S region were excluded because newly sequenced

species were invariable in this region and several existing GenBank accessions were missing the 5.8S region. For the rps16 intron comparative analysis, 27 new sequences were produced as part of this investigation and combined with 12 already published sequences (Downie & Katz-Downie, 1996) resulting in a rps16 data matrix of 39 taxa. New sequence data for the psbA-trnH cpDNA intergenic spacer were obtained for 24 taxa, and combined with two previously published sequences obtained from GenBank in a psbA-trnH data matrix of 26 taxa. Voucher specimen information and accession numbers are listed in Appendix 1. The plants identifications were based on published Floristic accounts and in comparison with verified herbarium specimens at E and on loan from other Herbaria (see Chapter 4).

8.2.3 DNA extraction, Amplification, and Sequencing

8.2.3.1 DNA extraction

Leaf material for DNA extraction was obtained either directly from living plants sampled in the field or from herbarium specimens. Total DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacture's protocol. Extracted DNA was used as a template for polymerase chain reaction (PCR) for ITS, rps16 and psbA-trnH sequencing.

8.2.3.2 Amplification

1. Primers

ITS region

PCR amplification of the ITS region, excluding the 5.8S rDNA region, was performed using primers ITS1 and ITS4 (White *et al.*, 1990). Additional primers ITS 5P (5' GGAAGGAGAAGTCGTAACAAG 3': manufactured by SIGMA), ITS 8P (5' CACGCTTCTCCAGACTACA 3': manufactured by TAGN) were used for this study, as primers ITS1 and ITS4 failed to generate PCR products for some samples.

Rps16 intron and psbA-trnH spacer region

The rps16 intron gene was amplified using PCR primers *rps16_F* (5' AAACGATGTGGTARAAAGCAAC 3': Shaw *et al.*, 2005) and *rps16_R* (5' AACATCWATTGCAASGATTCGATA 3': Shaw *et al.*, 2005).

psbA-trnH region

The psbA-trnH region was amplified with the primers *psbA_F* (5' GTTATGCATGAACGTAATGCTC 3') and *trnH_R* (5' CGCGCATGGTGGATTCACAAATC 3') described by Shaw *et al*. (2005).

Region	Primer	Direction	Sequence	Reference
ITS	ITS1P	Forward	GTAGGTGAACCTGCAGAAGGA	White *et al.*, 1990
	ITS5P	Forward	GGAAGGAGAAGTCGTAACAAG	Manufactured by SIGMA
	ITS4P	Reverse	TCCTCCGCTTATTGATATGC	White *et al.*, 1990
	ITS8P	Reverse	TGTAGTCTGGAGAAGCGTG	Manufactured by TAGN
rps16	rps16_F	Forward	AAACGATGTGGTARAAAGCAAC	Shaw *et al.,* 2005
	rps16_R	Reverse	AACATCWATTGCAASGATTCGATA	Shaw *et al.,* 2005
psbA-trnH	psbA_F	Forward	GTTATGCATGAACGTAATGCTC	Shaw *et al.,* 2005
	trnH_R	Reverse	CGCGCATGGTGGATTCACAAATC	Shaw *et al.*, 2005

2. PCR reaction conditions

PCR was performed using a MJ Research PTC-200 Thermal Cycler in 50$\mu\ell$ reaction volume. Apart from the primers, the PCR reagents used for amplification of ITS, rps16 and psbA-trnH were identical. The PCR reactions were performed in 50$\mu\ell$ using the following reagents:

Reagents	Quantity
10x Reaction buffer (10x: 160mM $(NH4)_2SO4$, 670mM Tris-HCL (pH 8.8 at 25℃) 0.1% Tween-20) (Bioline, London, UK)	5.0 $\mu\ell$
$MgCl_2$ (50mM) (Bioline, London, UK)	2.5 $\mu\ell$
dNTPs (0.2 mM) (Sigma Chemicals, Pool, Dorset, UK)	5.0 $\mu\ell$
forward primer (10 mM)	2 $\mu\ell$
reverse primer (10 mM)	2 $\mu\ell$
sterile, distilled water	31.5-32.5$\mu\ell$
DNA polymerase (5U/$\mu\ell$) (Bioline, London, UK)	0.125 $\mu\ell$
Extracted DNA sample	1-2 $\mu\ell$

The PCR cycling conditions used to amplify the ITS region were:

Temperature	Time	Number of cycles
94 ℃	3 min	x1
94 ℃	1min	x40
52 ℃	1min	
72 ℃	2min	
72 ℃	2min	x1

The PCR cycling conditions used to amplify the rps16 and psbA-trnH region were:

Temperature	Time	Number of cycles
94 ℃	3 min	x1
94 ℃	1min	x40
52 ℃	1min	
72 ℃	2min	
72 ℃	2min	x1

PCR amplification was assessed using 1% agarose gels (Agarose 1g, TBE (1%) 100ml, Ethydiumbromide 1$\mu\ell$). PCR products were purified using either Qiaquick MinElute PCR purification kit (Qiagen Ltd., Crawley, West Sussex, UK) or GFX PCR DNA Purification Kit (Amersham Biosciences, UK)

The purified products also were assessed on a 1% agarose gel.

8.2.3.3 Sequencing

Sequence amplification

Forward and reverse sequencing reactions were performed using the purified PCR products and were carried out in 10 μl reactions with the following compositions:

Reagents	(ITS)	(rps16)	(psbA-trnH)
Distilled sterile water	2-3 μl	2-3 μl	2-3 μl
DNA template	1-2 μl	1-2 μl	1-2 μl
Primer (10mM)	1 μl	1 μl	1 μl
DTCS Quickstart mix* (Dye Terminator Cycle Sequencing)	4 μl	4 μl	4 μl

*CEQ reagents from Beckmann Coulter Ltd., U.S.,

Sequence amplifications were achieved using a Perkin Elmer Thermo cycler PTC-200 PCR machine using the following conditions:

Temperature	Time	Number of cycles
96 ℃ 50 ℃ 60 ℃	2 min 20 sec 4 min	x 35

Sequence amplification product purification

Reactions were made up to 20 μl with distilled sterile water and transferred to a fresh 0.5 ml microfuge tube. To each tube 5 μl of stop solution (Stock B, see below) and 60 μl of 100 % ice cold ethanol were added.

Stock B	Volume per reaction
0.5M EDTA (Beckmann Coutler Ltd., UK)	0.4 μl
Sigma water (Beckmann Coutler Ltd., UK)	1.6 μl
3M NaOAc pH 5.2 (Beckmann Coutler Ltd., UK)	2 μl
Glycogen (Beckmann Coutler Ltd., UK)	1 μl

The sample were mixed using a vortex mixer and centrifuged for 15 minutes at 4 ℃ at 14,000 rpm. The supernatant was carefully removed and discarded. 200$\mu\ell$ of 70% ice cold ethanol was added, and the samples centrifuged at 4℃ at 14,000 rpm. The supernatant was again carefully removed and discarded. The last step was repeated and the supernatant was removed and discarded again. The remaining pellet was vacuum dried on a low drying rate for 2 to 5 minute until no trace of ethanol remained. Each pellet was then resuspended in 40$\mu\ell$ of sample loading solution (SLS) (Beckmann Counter Ltd., UK) and mixed using a vortex mixer. Samples were stored at -20℃ for storage. All sequences were read on an ABI Prism™ 377 DNA Sequencer, and then edited manually using Sequence Navigator 1.0 (Applied Biosystems Inc.), which were read on a Beckmann Coulter CEQ™ 8000 Analysis System DNA Sequencer (Beckmann Counter Ltd., UK).

Analysed sequences (Forward and reverse) were edited using CEQ™8000 Genetic Analysis System Version 7.0 Software (Beckmann Counter Ltd., US) and aligned in Sequencher™ Version 4.5 (Genetic Codes Corporation, Ann Arbor, Michigan, US). At least two reads (forward and reverse sequences) were used to generate each consensus. The contig sequences were exported as text files for phylogenetic analysis. Sequeces obtained from the Sequencher were BLAST (Basic Local Alignment Search Tool) searched in GenBank (NCBI: National Centre of Biotechnology Information). Results indicated that some obtained sequences were from fungal contaminats

8.2.4 Sequence results and alignment

All sequences were aligned manually in the data editor of PAUP* version 4.0β (Swofford, 2001), with gaps positioned to minimize nucleotide mismatches. Indels of more than one base were coded as the same presence/absence character state if they could not be interpreted as separate duplication or insertion events. In several regions, gap coding was problematic because of homopolymers or indirect duplications of adjacent elements in two or more taxa. These gaps were not scored and these ambiguous regions were excluded from subsequent analyses. Boundaries of the genes ITS, rps16, and psbA-trnH were determined by comparison of the DNA sequences to corresponding boundaries in other Apiaceae (ITS & rps16: Downie *et al*., 2000a, b; psbA-trnH: Kress *et al*., 2005). The nucleotide sequence data reported in this study have been deposited in the GenBank Library (ITS and rps16); accession numbers are provided the Table Appendix 1.

8.2.5 Phylogenetic analysis of molecular data

Three sets of phylogenetic analyses were undertaken. Initially, a maximum parsimony analysis of ITS data for all 64 taxa was carried out to test monophyly of the '*Heracleum* clade' and placement of Sino-Himalayan members of *Heracleum* and related genera. The second analysis of a smaller matrix from the rps16 intron used maximum parsimony separately (39 taxa) and in combination with ITS data to test the robustness of the ITS result.

To examine the extent of conflict among separate data sets, the incongruence length difference test (Farris *et al*., 1994) was conducted using the partition

homogeneity test within PAUP*. One hundred replicates were considered for each partition, using simple addition sequence of taxa and tree bisection reconnection (TBR) branch swapping. Incongruence among data sets is identified if the additive tree lengths taken from resampled matrices are greater than the sum of the tree lengths from the original data.

Nucleotide sequences were analysed using parsimony. Analyses were performed using PAUP* 4.0β10 (Swofford, 2001). Descriptive tree statistics were given by the consistency index (CI), homoplasy index (HI), and retention index (RI) (Farris, 1989). All trees were obtained from unweighted, unordered characters. Multistate characters were interpreted as uncertain and gaps were treated as missing. For character optimisation the option ‘accelerated transformation (ACCTRAN)’ was used, which favours reversals. Starting trees were obtained via stepwise addition. Heuristic search options included an initial heuristic search using 10,000 random addition replicates, tree-bisection-reconnection (TBR) branch swapping with steepest descent off and Multrees activated. Trees produced from this analysis were used as the basis for a further heuristic search, activating steepest descent and Multrees and saving 10,000 trees which are considered to be sufficient to capture all topological variation. To limit the number of trees found, the “collapse branches if minimum length is 0” option was implemented. As an approximate guide to clade support, bootstrap values (Felsenstein, 1985) were calculated for the three combinations of analyses using 10,000 bootstrap replicates each heuristic search on one random addition replicate, and TBR branch swapping. Steepest decent and Multrees were not activated.

Bayesian inference of the separate and combined ITS, rps16 intron, and psbA-trnH were conducted using MrBayes vers. 3.1.1 (Huelsenbeck & Ronquist, 2001). Prior to analysis, Modeltest 3.6 (Posada & Crandall, 1998) was used to select evolutionary model of nucleotide substitution, is given in Table 8.3, that best fits each partition in the data as selected by the Akaike Information Criterion estimator. Parameters utilized for the BA are shown in Table 8.5. The settings appropriate for the best-fit models were put into an MrBayes block in PAUP*. From different random starting trees, four independent Bayesian analysis (nruns=4) were run for 10 million generations and the trees saved to a file every 1000 generations. Four simultaneous Markov Chain Monte Carlo (MCMC) chains were used and branch lengths of the trees were saved. The states of the chain that were sampled before stationarity (i.e., the “burn in” of the chain) were discarded (25% proportion discarded after checking stationarity) and the posterior probability values for each bipartition of the phylogeny were determined from the remaining trees. Phylogenetic trees were generated as phylograms, strict consensus and majority rule consensus trees.

8.3 Results

8.3.1 Sequence analysis (Table 8.1)

8.3.1.1 ITS

65 accessions examined for ITS sequence variation, the length of the ITS 1 and ITS 2 region combined varied from 420 to 443 base pairs (bp). Data from the intervening 5.8S region was excluded because newly sequenced species were invariable in this region and several existing GenBank accessions were missing this region. The alignment of complete ITS sequences resulted in a matrix of 616 positions. Of these, 197 were excluded from subsequent analysis because of alignment ambiguities (see Sequence Alignments above) resulting in a matrix of 419 unambiguously aligned positions. The ambiguous positions represented tracts of poly-As, -Gs or –Ts, or indirect duplications of adjacent elements in two or more taxa. Characteristics of all unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 8.3. Unambiguous alignment gaps were potentially parsimony informative, ranged from two to three base pairs in size. Pairwise sequence divergence ranged from 0 (identical) to 18 % of nucleotides within the ingroup, and ranged from 11.3% to 24.3% between the outgroup and ingroup respectively.

8.3.1.2 Rps16

The rps16 intron sequence in the 39 species examined varied from 783 to 878 bp. The alignment of the 39 complete rps16 intron sequences resulted in a matrix of 966 positions. 93 positions were excluded from further consideration due to ambiguity of alignment (as above). Characteristics of all unambiguous positions,

including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 8.1. Sixteen unambiguous alignment gaps were potentially parsimony informative, and ranged from five to 22 base pairs in size. Pairwise sequence divergence ranged from 0 (identity) to 2.85 % of nucleotides within the ingroup, and ranged from 1.03% to 4.49% of nucleotide between the outgroup and ingroup respectively.

8.3.1.3 psbA-trnH

The psbA-trnH sequence in the 26 species examined varied from 277 to 343 bp. The alignment of the 26 complete psbA-trnH sequences resulted in a matrix of 405 positions. 101 positions were excluded from further consideration due to ambiguity of alignment. The characteristics of all the unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in table 8.1. 17 unambiguous indels were introduced and potentially parsimony informative, ranging from three to 26 base pairs in size. Pairwise sequence divergence ranged from 0 (identity) to 3.2 % of nucleotide within the ingroup and ranged from 3.65% to 4.36% of nucleotides between the outgroup and ingroup respectively.

8.3.1.4. ITS and rps16 intron combined data

ITS and rps16 intron data for the same set of 39 taxa were combined for simultaneous consideration. The partition homogeneity test between the ITS and rps16 data sets gave a value of P=0.129, reflecting no significant incongruence between data sets. Similarly, a review of bootstrap support values between the separate ITS and rps16 intron topologies showed no incompatible clades (except

Angelica clade, See Fig. 8.10) regardless of the bootstrap support. It was therefore deemed acceptable to combine both these data sets. The sequences for the 39 taxa in the ITS and rps16 intron combined data set varied from 1203 to 1321 bp. The alignment of 39 complete combined sequences resulted in a matrix of 1582 positions. Here, 290 positions were excluded from further consideration due to ambiguity of alignment. Characteristics of all unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 8.1.

8.3.1.5. ITS, rps16 intron and psbA-trnH combined analysis

ITS, rps16 intron and psbA-trnH data for the same set of 26 taxa were combined for simultaneous consideration. The partition homogeneity test between the ITS and rps16 data sets gave a value of P=1.0 between ITS and psbA-trnH data sets gave a value of P=0.849, and rps16 and psbA-trnH data sets gave a value of P=0.193 reflecting no significant incongruence between data sets, therefore, the three data sets were combined.

The sequences for the 26 species in the three-way combined data set varied from 1480 to 1664 bp. The alignment of 26 complete combined sequences resulted in a matrix of 1987 positions. Here, 391 positions were excluded from further consideration due to ambiguity of alignment. Characteristics of all unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 8.1

8. 3. 2 Phylogenetic analysis

8.3.2.1. ITS

Maximum Parsimony (Table 8.2)

Maximum parsimony (MP) analysis of all 419 unambiguously aligned positions resulted in 128 most parsimonious trees, each 584 steps long, with consistency indices of 0.611 (0.521 without uninformative characters), and a retention index of 0.793. The strict consensus of these trees is not fully resolved, but shows better resolution when gap characters were included in the analysis (Fig. 8.1). Some branch support values were also increased as a result.

In the strict consensus tree the seven accessions (*Anethum*, *Angelica, Ammi, Apium, and Conium*) comprise the outgroup to the other clades. The remaining genera, *Heracleum*, *Pastinaca*, *Malabaila*, *Zosima*, *Tordylium*, *Semenovia*, and *Tordyliopsis* comprise a strongly supported monophyletic group (98% bootstrap, 1.0 posterior probability). This result support previous works (Katz-Downie *et al*., 1999; Downie *et al*., 2000a & b; Downie *et al*., 2001) where this group has been recognized as the '*Heracleum* clade'.

Within the '*Heracleum* clade' six distinct clades are recognized, Fig. 8.1, that correspond to approximate generic groups with several unresolved taxa (e.g. *Semenovia*, *Tordylium*, *Tordyliopsis*, and *Heracleum millefolium*). *Heracleum* is included in at least two distinct clades, so it does not appear to be monophyletic. *Heracleum* species are widely distributed throughout the tree and within separate lineages A1, A2, A3. Each clade has relatively low bootstrap value (A1 63%, A2

74%, A3 77%), however, posterior probabilities of each clade (Fig. 8.2) are high (0.97 pp average).

The clade with the largest number of taxa (Clade A1) is not strongly supported (Fig. 8.1, 63% bootstrap). *Heracleum austriacum* is sister to all the other species in Clade A1. Without *H. austriacum*, this group is more highly supported (100% bootstrap). Within A1, three moderately to well-supported sub-clades (B1-B3) are recognized, all being a sister to *H. austriacum*.

Sub-clades B1 and B3 have high branch support values (87% and 99% bootstrap respectively) and Clade B2 has moderate support value (54% bootstrap). Sub-clade B1 consists of *Heracleum scabridum*, *H. franchetii*, *H. stenopterum* and *H. forrestii* complex. (bootstrap 83%). Sub-clade B2 is not strongly supported and contains unresolved species (bootstrap 54%). *H. mantegazzianum*, and its hybrid with *H. sphondylium*, is sister to the *H. moellendorfii* complex. Sub-clade B3 consists of *H. sphondylium* and *H. dissectum* as sister to all other sub-clades (bootstrap 99%, posterior probability 1.0).

Clade A2 is moderately supported by 74% bootstrap. This Group is always subdivided into two sub-clades in all analysis. Clade A3 (*Heracleum* Group III; 77% bootstrap) is not resolved very well, and falls on a polytomy which contains *H. candicans* and *H. obtusifolium*. The genera *Pastinaca* and *Malabaila* comprise a strongly supported monophyletic group Clade C (99% bootstrap). *Pastinaca* and *Malabaila* are not separated in the analyses, and *P. armena* and *M. involucrata* are not resolved. This clade forms the sister to Clade A1 (*Heracleum* Group I). All representative species of *Zosima* (Clade D: *Z. absinthifolia*, *Z. radians*, and *Z.*

orientalis) comprise a well supported monophyletic group (99% bootstrap, 1.0 posterior probability).

The MP analysis goes some way towards resolving the taxonomic placement of morphologically and taxonomically enigmatic taxa such as *Tordyliopsis brunonis*, *Heracleum millefolium*, *Semenovia* species and *Tordylium* species. The two *Heracleum millefolium* accessions are not included in any of the other *Heracleum* groups, but form a separate, well supported clade (100% bootstrap). The monotypic genus *Tordyliopsis* is not resolved with regard to other clades A2 and A3, but is always recognized as an isolated branch. The species of *Semenovia* are not resolved. The two *Tordylium* taxa included in the analysis, unexpectedly appear in different places within the 'Major *Heracleum* clade' (give code letter/number) although neither is related to of the other clades recognised. The exclusion of these two taxa from the analysis did not affect topology.

Bayesian analysis

The topology of the Bayesian AIC majority rule consensus tree, calculated using a GTR+G model, is given in Table 8.3. It is almost identical to the MP strict consensus tree. The posterior probability values (pp) for the different clades were generally higher than the bootstrap supports. The tree topology based on hLRTs was fully congruent, with similar pp values.

Only a few areas of the Bayesian (BA) tree were not congruent with the MP strict consensus tree. The main difference was that *H. mantegazzianum* 1 and *H. mantegazzianum* 2 were no longer nested within clade B2. Instead, these species

are resolved as sister to clades B1, B2 and B3. In the MP analysis, clade A2 is not strongly supported, but in the BA analysis Clade A2 was more strongly supported (pp 0.92, 1.00), though resolution within this clade was less clear. The *Heracleum* clade A3 forms a much more strongly supported clade (pp 1.00) compare to MP analysis. The relation within clade C is now fully resolved, and *Pastinaca* and *Malabaila* are split into two separate groups. The relation between the *Zosima* species is also now fully resolved in the BA analysis, and *Tordylium elegans* is inferred to be sister to the *Zosima* group (group D), although this is only weakly supported. *Heracleum cachemericum* and *Semenovia* species, which are weakly grouped together in the MP analysis (<50% bootstrap), are now unresolved in BA analysis. All the enigmatic taxa mentioned above are similarly placed in the BA analysis. *Heracleum millefolium* accessions again form a distinctive and separate clade which is very well supported (1.0 posterior probability). *Tordyliopsis* is also recognized on its own isolated branch. The species of *Semenovia* are not included within any other group, and the two *Tordylium* species appear to be sister to group A1 and C.

8.3.2.2 Rps16

Maximum parsimony (Table 8.2)

Maximum parsimony analysis of 878 unambiguously aligned rps 16 intron nucleotides plus nine binary-scored informative gaps, with gap states treated as missing data, resulted in six most parsimonious trees. Each trees was 151 steps long, with consistency indices of 0.781 and 0.467 (without uninformative characters), and a retention index of 0.851. The strict consensus tree (with accompanying bootstrap values) is shown in Fig. 8.4. Bootstrap values were

between 58 to 100%. The strict consensus of these trees is not fully resolved and comprises many polytomies, however, better resolution is achieved when gap characters were included in the analysis. Some branch support values are also increased as a result. This strict consensus tree is generally congruent to those obtained in the analyses using a broader sampling of ITS nucleotides. In the strict consensus tree four included accessions (*Conium*, *Apium*, *Ammi*, and *Anethum*) comprise out groups to the other clades, However, it is interesting to note that in this analysis the *Angelica* species group (92% bootstrap), which formed the out group in the ITS analysis, is no longer out group of '*Heracleum* clade'. It appears to form a close sister group to other clades. With the *Angelica* clade, the genera *Heracleum*, *Pastinaca*, *Malabaila*, *Zosima*, *Tordylium*, *Semenovia*, and *Tordyliopsis* comprise a strongly supported monophyletic group (100% bootstrap, 100% posterior probability). Within the main '*Heracleum* clade' *Heracleum* species are again widely distributed throughout the tree and form three main separate lineages (Group A1, A2, A3), which correspond to those obtained in the ITS analysis. *Heracleum* is again shown not to be monophyletic.

Branch support value for Group A1 is higher than in the ITS analysis (78%, bootstrap, 100% posterior probability). Group A1 fell on a polytomy which also contains *H. austriacum* as sister to all the other speices. *Pastinaca* and *Malabaila* species are here sister to group A1 and moderately supported (77% bootstrap, 100% posterior probability). Group A2 is recognizable, albeit poorly supported (bootstrap <50%), however, in the Bayesian anlysis (Fig. 8.5), this group was very well supported (100% posterior probability). Group A3 comprised of *Heracleum candicans* and *H. obtusifolium* is highly supported (Group D, ADD bootstrap and

pp??). Representative species of *Zosima*, which are highly supported as a group in the ITS analysis (99% bootstrap, 100% posterior probability) are not well resolved here. The placement of the enigmatic taxa was very similar to that in the ITS trees (Fig. 1, 2) The only differences are that *Semenovia dasycarpa* is placed in a group with *Heracleum cachemericum*, and *Tordylium elegans* arises as sister to Group A1 and Group C.

Bayesian analysis (Fig. 5)

The Bayesian majority rule consensus tree is mostly congruent to the MP strict consensus tree. The posterior probability values (pp) for the different groups were generally higher than the bootstrap supports, and ranged from 0.57-1.00. Only a few areas of the tree were not congruent with the MP strict consensus tree. In the MP analysis Group A2 is poorly supported (bootstrap <50%), however, in the Bayesian anlysis, this group was well supported (1.0 posterior probability). Inside this group two well-supported sub-group (*H. nepalense* & *H. sublineare*: 0.83; *H. hookerianum* & *H. rigense*: 0.92) are recognized.

8.3.2.3 psbA-trnH

Maximum parsimony (Table 8.2)

Maximum parsimony analysis of 322 unambiguously aligned psbA-trnH nucleotide plus nine binary-scored informative gaps, with gap states treated as missing data, resulted in six most parsimonious trees, each 34 steps long, with consistency indices of 0.912 and 0.788 (without uninformative characters), and a retention index of 0.968. Bootstrap branch support values ranged from 60 to 100%.

The strict consensus trees of psbA-trnH is not well resolved with most of the ingroup taxa comprising a large polytomy (Fig. 8.6.A). The topology of psbA-trnH is unfortunately not congruent with the other two DNA markers studied. Although this cladogram failed to resolve many of the relationships, at least, it does strongly support the monophyly of the 'Major *Heracleum* clade' (100% bootstrap). In the strict consensus tree two included accessions (*Conium* and *Foeniculum*) comprise out groups to the other groups. The only strongly supported group within the 'Major *Heracleum* Clade' is Group A1 + Group C (*Pastinaca-Malabiala*) group (94% bootstrap). Sub-group B1 is also recognized and moderately supported.

Bayesian analysis

The Baysiean tree, calculated using K81uf model (Fig. 8.6.B), is, like the MP consensus tree, not fully resolved, but does have a similar topology. Again only Group A1 + Group C (*Pastinaca-Malabiala*) group form a highly supported (1.0 posterior probability) separate group. Within this group two sub groups are recognized. *H. mantegazzianum* and *H. lanatum* are also recognized with 0.81. Sub-group B1 also recognized (93%) consisting exclusively of Chinese *Heracleum* species from Yunnan province.

Combined analysis

8.3.2.4 ITS and rps16 intron combined data

Maximum parsimony (Table 8.2)

The parsimony analysis of the 1310 parsimony informative characters from the combined ITS and rps16 intron sequences yielded 343 most parsimonious trees with a tree length of 667, a consistency index of 0.663 (0.484 excluding uninformative characters) and a retention index of 0.764. Their strict consensus is presented in Fig 8.7 and a single representative tree in Fig. 8.8. Identical results were obtained when gap states were treated as a fifth base; slightly less resolution was achieved when gap positions were excluded from the analysis. The strict consensus tree shows identical topologies in general compared with the ITS strict consensus tree (Fig. 8.11), and greater bootstrap and posterior probability support for many of the groups recognized in the ITS and rps16 analyses alone. In the strict consensus tree *Angelica* species form a well differentiated group and firmly part of the out group, but retains a close sister relationship to the monophyletic 'Major *Heracleum* clade'. Within the main clade, *Heracleum* species are again widely distributed throughout the tree and forms three separate main lineages (Group A1, A2, and A3), thus *Heracleum* is again shown to be not monophyletic.

The branch support value for Group A1 is higher than in the separate ITS and rps16 analyses (93%, bootstrap, 100% posterior probability), and the relationships of taxa within Group A1 is more resolved. *Heracleum austriacum* as sister to all the other species. Branch support value for Group C (*Pastinaca* and *Malabaila* group) is again higher than in the separate ITS and rps16 analyses (100%, bootstrap, 100% posterior probability). Within Group C the relationships of

species between the two genera are clearly resolved, but the placement of *Pastinaca armena* is still ambiguous. Groups A2, A3 and enigmatic taxa such as *Tordyliopsis* and *Semenovia* form weakly supported group. But Group A2 + Group A3 is highly supported (82% bootstrap, 100% bootstrap). The monotypic genus *Tordyliopsis* is placed sister to *Group A2*, although this is very poorly supported. Representative species of *Zosima*, which not supported in the rps16 analysis, now form a well supported group (99% bootstrap, 100% posterior probability). *Heracleum millefolium* is again recognized on an isolated branch. *Tordylium elegans* is placed sister to Group A1+ Group, as in ITS and rps16 analyses. The remaining species of *Semenovia* and *Heracleum cachemiricum* are not resolved and placed sister to other groups.

Bayesian analysis

The topology of the majority rule consensus tree of the Bayesian analysis (Fig. 8.9) was nearly identical to the MP strict consensus tree, and only differed in minor areas. The posterior probability values (pp) for the different groups were generally higher than the MP bootstrap supports, and ranged from 0.60-1.00. Group A1 is here highly supported (pp 1.00) and most species within this group, which were formerly resting on a polytomy in the MP tree, are better resolved with higher group credibility. The relationships within Group C, which forms sister to group A1, are here fully resolved. *Pastinaca* and *Malabaila* are separated into two separate groups, although the placement of *Pastinaca armena* was weakly supported. *Tordylium elegans* is here sister to the *Heracleum* A1 and Group C with high credibility (pp 1.00). Group A2 formerly rested on a polytomy with weak support (bootstrap <50%) in the MP analysis, but in the BA analysis it forms a

more resolved group with high group credibility values (pp 1.00). Zosima is well supported (pp 1.00) and the species of *Semenovia* and *Heracleum cachemericum* now form a weakly supported group (pp 0.57). Group A3 (*H. candicans* & *H. obtusifolium*) is strongly supported (pp 1.0) with close relationship with *Tordyliopsis brunonis* (pp 0.82*)*. *Heracleum millefolium* forms a basal clade of group A2, A3, D, and remaining species (*Semenovia*, *Tordyliopsis*).

8.3.2.5 ITS, rps16 and psbA-trnH combined analysis

Maximum parsimony (Table 8.2)

Maximum parsimony analysis of 1635 unambiguously aligned combined sequences with binary-scored informative gaps, with gap states treated as missing data, resulted in 33 most parsimonious trees, each 492 steps long, with consistency indices of 0.736 and 0.503 (without uninformative characters), and a retention index of 0.766, of which their strict consensus (with accompanying bootstrap values) is shown in Fig. 8.12. Bootstrap branch support values were between 60 to 100%. To show better resolution gap characters were included in the analysis. Some branch support values were also increased as a result.

The all combined strict consensus trees of ITS, rps16 and psbA-trnH is fairly well resolved. *Conium*, and *Foeniculum* comprise outgroups to the other groups. Ingroup taxa, which are generally resting on two groups, comprise a strongly supported monophyletic group (100% bootstrap). Within the group, *Heracleum* species are widely distributed throughout the tree and form mainly three separate lineages (Groups A1, A2, A3), thus *Heracleum* is again confirmed as not

monophyletic. Branch support value for Group A1 is higher than in the separate ITS and rps16 analyses (88%, bootstrap, 100% posterior probability). Also the relationships of taxa within the Group A1 was more fully resolved, and *H. austriacum* is confirmed as sister to all the other species. Branch support value for the *Pastinaca* and *Malabaila* group (Group C) is higher than in the ITS, rps16 or ITS + rps16 combined analyses (100%, bootstrap, 100% posterior probability). Group A2 is less well supported than ITS + rps16 combined sequences (64% bootstrap, 100% posterior probability). Group A3 (*Heracleum candicans* and *H. obtusifolium*) is highly supported (100% bootstrap, 100% posterior probability), and *Zosima absinthifolia* and *Z. radians* form a highly supported Group D (100% bootstrap, 100% posterior probability). The placement of the enigmatic genus *Tordyliopsis* and *H. millefolium* is not resolved with respect to the other species, but again form isolated branches. The remaining species of *Semenovia* and *Heracleum cachemiricum* are again not resolved here and fall on a polytomy.

Bayesian analysis

The topology of the Bayesian majority rule consensus tree (Fig. 8.13) is almost congruent to the MP strict consensus tree. The posterior probability values (pp) for the different groups were generally higher than the bootstrap supports. The main difference was that BA tree forms a fully resolved topology. Group A2 is here highly supported (pp 1.00). Most species within this group, which were formerly resting on a polytomy in the MP analysis, are better resolved with higher credibility. Group A3 forms a well supported group (pp 1.00) as sister to *Tordyliopsis brunonis* (pp 0.64). *Heracleum millefolium* is here sister to the *Zosima* group (Group D) although this is only weakly supported (pp 0.73).

Semenovia group (*Heracleum cachemericum*, *Semenovia dasycarpa* and *Semenovia transiliensis*), which are not resolved in MP analysis, here form a highly supported group (pp 0.99).

Table 8.1 Sequence characteristics of ITS and rps16, and psbA-trnH regions, separately and combined, used in the phylogenetic analysis of *Heracleum* and related genera.

					Combined	
Parameter	**Program**	**ITS**	**rps16**	**psbA-trnH**	**ITS+rps16**	**ITS+rps16+psbA-trnH**
Length range (total) (bp)	PAUP	420-443	783-878	277-343	1203-1321	1480-1664
Length mean (total) (bp)	PAUP	436.92	853.74	321.58	1290.66	1612.24
Length range (ingroup) (bp)	PAUP	420-443	783-878	288-343	1203-1321	1491-1664
Length mean (ingroup) (bp)	PAUP	436.9	849.03	322.83	1285.93	1608.76
Length range (outgroup) (bp)	PAUP	435-440	862-883	277-336	1297-1323	1574-1659
Length mean (outgroup) (bp)	PAUP	437.14	875.29	306.5	1312.43	1618.93
Aligned length (bp)	PAUP	616	966	405	1582	1987
Number of excluded site	PAUP	197	93	101	290	391
Sequence divergence (ingroup) (%)*	PAUP	0-18.0	0-2.85	0-3.20	0-18.0	0.18.0
Sequecne divergence (in/outgroup) (%)*	PAUP	11.3-24.3	1.03-4.49	3.65-4.36	1.03-24.3	1.03-24.3
Number of parsimony informative indels (ingroup)*	PAUP	3	9	8	12	20
Number of parsimony informative indels (total)*	PAUP	6	16	17	22	39
Size of indels (ingroup)*	PAUP	2-3	5-22	3-17	2-22	2-22
Size of indels (total)*	PAUP	2-3	5-22	3-26	2-22	2-26
Number of sites after exclusion*	PAUP	419	878	313	1297	1610
Number of variable sites*	PAUP	253	113	22	366	388
Number of constant sites (%)*	PAUP	166 (39.6)	765(87.1)	291(92.9)	931	1222
Number of informative sites (%)*	PAUP	163(38.9)	56	10	219	229
Number of autapomorphic sites (%)*	PAUP	90	57	12	147	159

*Based on alignment excluding ambiguous sequence sites

Table 8.2. The result of the parsimony analysis

		Characters included in Parsimony analysis	No. of characters in analysis	No. of best trees	Length of best tree	Consistency index (CI)	Retention index (RI)	Rescaled consistency index (RC)	Homoplasy index (HI)
ITS (65)		All characters except ambiguous	419	128	584	0.611/0.521*	0.793	0.484	0.389
rps16 intron (39)		All characters except ambiguous	878	6	151	0.781/0.467*	0.851	0.665	0.219
psbA-trnH (26)		All characters except ambiguous	322	6	34	0.912/0.788*	0.968	0.882	0.088
Combined	**ITS + rps16 (39)**	All characters except ambiguous	1310	343	667	0.663/0.484*	0.764	0.506	0.337
	ITS + psbA-trnH (26)	All characters except ambiguous	750	4	387	0.708/0.503*	0.729	0.516	0.292
	rps16 + psbA-trnH (26)	All characters except ambiguous	1021	2	137	0.869/0.503*	0.922	0.801	0.131
	ITS+ rps16+ psbA-trnH (26)	All characters except ambiguous	1635	33	492	0.736/0.503*	0.766	0.564	0.264

* Consistency indices (CI) based on informative characters only; ( ): number of taxa

Table 8.3 Model selection and parameter values for Bayesian analysis

Based on the Akaike information Criterion (AIC) and the hLRTs (hierachical Likelihood Ratio Tests) in the program Modeltest (Posada & Crandall, 1988), ( ) : number of taxa

Partition			**Model**	**-ln likelihood**	**Parameter values**
ITS			GTR+G	3699.6047	nst=6 rates=invgamma, Shapepr=0.6309, Pinvarpr=0, Statefreqpr=(0.25, 0.25, 0.25, 0.25) Revmatpr=(0.5444, 2.4812, 1.4255, 0.5487, 5.5600, 1)
rps16 intron			TVM+I+G	2180.9338	nst=6 rates=gamma, Shapepr=1.0004, Pinvarpr=0.4692, Statefreqpr=(0.3627, 0.1357, 0.1668, 0.3348) Revmatpr=fixed(0.6336, 0.7436, 0.1756, 0.0374, 0.7436, 1)
psbA-trnH			K81uf	533.7531	nst=6 rates=equal, Pinvarpr=0, Statefreqpr=fixed(0.3678, 0.1281, 0.1576, 0.3465) Revmatpr=fixed(1.0000, 0.9969, 0.3063, 0.3063, 0.9969, 1)
Combined	**ITS + rps16**	**ITS (39)**	SYM+G	3164.3945	nst=6 rates=gamma, Shapepr=0.8256, Pinvarpr=0, Statefreqpr=(0.25, 0.25, 0.25, 0.25) Revmatpr=(0.5413, 2.5990, 1.7076, 0.5901, 5.0667, 1)
		Rps16 (39)	TVM+I+G	2180.9338	nst=6 rates=gamma, Shapepr=1.0004, Pinvarpr=0.4692, Statefreqpr=(0.3627, 0.1357, 0.1668, 0.3348) Revmatpr=fixed(0.6336, 0.7436, 0.1756, 0.0374, 0.7436, 1)
	ITS + psbA-trnH	**ITS (26)**	SYM+G	2442.4819	nst=6 rates=gamma, Shapepr=0.7006, Pinvarpr=0, Statefreqpr=fixed(0.2419, 0.2238, 0.2420, 0.2923) Revmatpr=fixed(1.0000, 3.2787, 1.0000, 1.0000, 6.5729, 1)
		psbA-trnH (26)	K81uf	533.7531	nst=6 rates=equal, Pinvarpr=0, Statefreqpr=fixed(0.3678, 0.1281, 0.1576, 0.3465) Revmatpr=fixed(1.0000, 0.9969, 0.3063, 0.3063, 0.9969, 1)
	rps16 + psbA-trnH	**Rps16 (26)**	TVM+G	1744.5278	nst=6 rates=gamma, Shapepr=0.3313, Pinvarpr=0, Statefreqpr=fixed(0.3556, 0.1361, 0.1677, 0.3406) Revmatpr=fixed(0.4484, 0.5498, 0.1098, 0.0486, 0.5498, 1)
		psbA-trnH (26)	K81uf	533.7531	nst=6 rates=equal, Pinvarpr=0, Statefreqpr=fixed(0.3678, 0.1281, 0.1576, 0.3465) Revmatpr=fixed(1.0000, 0.9969, 0.3063, 0.3063, 0.9969, 1)
	ITS+ rps16+ psbA-trnH	**ITS (26)**	SYM+G	2442.4819	nst=6 rates=gamma, Shapepr=0.7006, Pinvarpr=0, Statefreqpr=fixed(0.2419, 0.2238, 0.2420, 0.2923) Revmatpr=fixed(1.0000, 3.2787, 1.0000, 1.0000, 6.5729, 1)
		Rps16 (26)	TVM+G	1744.5278	nst=6 rates=gamma, Shapepr=0.3313, Pinvarpr=0, Statefreqpr=fixed(0.3556, 0.1361, 0.1677, 0.3406) Revmatpr=fixed(0.4484, 0.5498, 0.1098, 0.0486, 0.5498, 1)
		psbA-trnH (26)	K81uf	533.7531	nst=6 rates=equal, Pinvarpr=0, Statefreqpr=fixed(0.3678, 0.1281, 0.1576, 0.3465) Revmatpr=fixed(1.0000, 0.9969, 0.3063, 0.3063, 0.9969, 1)

8.4 DISCUSSION

8.4.1 Sampling

Central Himalayan species of *Heracleum* are well covered, but only 13 of a possible 27 species from SW China (Pu & Watson, 2005) could be included. A major problem for molecular studies is that many *Heracleum* species recorded from SW China are only known from old historical collections which are in very poor condition and too old for molecular study. Many of these old specimens were sampled in this study, but the DNA was found to be too degraded and unusable. Another problem is that many of these older specimens are incomplete and not representative of these large plants and so accurate species identification is often ambiguous. Therefore, Chinese taxa had to be carefully selected to only include those with unequivocal identification and material with DNA in good enough condition for sequencing. There were similar problems with *Heracleum* species from Southern India, SE Asia (Myanmar and Thailand) as these are only known from very old material and again many DNA samples were taken from these herbarium specimens but this was found to be too degraded to use. Although fieldwork was undertaken to parts of SW China (see Chapter 4) to study population level variation and to collect fresh herbarium and DNA material, an extensive series of expeditions would be required to complete the sampling in SW China and the other areas in Asia.

8.4.2 Monophyly of the 'Major *Heracleum* clade' *sensu* Downie

The '*Heracleum* Clade' *sensu* Downie has previously been identified within the Apioid super clade to comprise *Heracleum*, *Pastinaca*, *Malabaila*, *Tetrataenium*, *Zosima*, *and Tordylium* (Katz-Downie *et al*., 1999; Downie *et al*., 2000a & b; Downie

et al., 2001). These high-level studies outlined the content of the '*Heracleum* Clade', but were limited by sampling (10 species) and were considered provisional as the rps16 analysis results did not fully support the monophyly of this clade as there problems with an equivocal positioning of *Tordylium* (Downie *et al*., 2000a). The present study, with a much enlarged taxon sampling and data from three gene regions, confirms the monophyly of the previous circumscription of the '*Heracleum* Clade' (Figs. 8.1, 8.2) and extends the circumscription to include *Semenovia* and *Tordyliopsis.* This clade is very strongly supported by MP (93 -100% bootstrap support) and BA analysis (100% posterior probability). The result from the present study, the combined and separate analysis of ITS, rps16, and psbA-trnH, have confirmed that *Heracleum* clade is monophyletic. However, in this study psbA-trnH spacer failed to resolve relationships among the *Heracleum* clade although the utility of chloroplast region psbA-trnH has been successively tested in other plant families (Kress *et al.*, 2005). In this analysis of psbA-trnH region was rather conservative, and this spacer seems to be less variable than either ITS and rps16. The present study shows that '*Heracleum* Clade', with the addition of *Pastinaca*, *Semenovia* and *Tordyliopsis*, corresponds to Tribe Tordylieae W.D.J. Koch (=Drude's Peucedaneae Subtribe Tordyliinae). It includes eight of the 23 genera within Tribe Tordylieae as delimited by Pimenov & Leonov (1993). Monophyly of Tribe Tordylieae and its separation from other Peucedanoid taxa has been previously supported by Shneyer *et al*. (1995), using seed-protein immunochemistry.

Conium maculatum, *Apium graveolens*, *Ammi majus*, *Anethum graveolens*, and *Angelica* species are used for the outgroup. All present separate and combined studies (except rps16 intron) support their sister status or outgroup to the 'Major

Heracleum clade'. While rps16 intron data from the present study study suggests that genus *Angelica* (*A. ampla*, *A. pinnata*, *A. breweri*) is included in the '*Heracleum* clade', the separate analysis of ITS and combined analysis of ITS + rps16 do not include *Angelica* within the '*Heracleum* clade'. Drude's treatment (1897-98) recognized three subtribes within Peucedaneae on the basis of morphology of the fruit wings. *Angelica* was included within Angelicinae characterized by lateral wings which separate during maturation. *Pastinaca* and *Heracleum* were included in Ferulinae (Peucedaninae) and Tordyliinae respectively and characterized by closely appressed lateral wings and thickened wing margins. Thus fruit morphology agrees with the close relationship of *Angelica* to *Heracleum* and *Pastinaca*, and also supports this separation.

A very recently study of SW Asian and European Apiaceae (Yousef *et al*., In press) includes further sampling from the '*Heracleum* clade' and agrees with the results of the present study, with the additional inclusion of European species of *Heracleum*, *Semenovia*, and *Ainsworthia* within the expanded '*Heracleum* Clade' which is now formally equated with Tribe Tordylieae.

8.4.3 Polyphyly of Sino-Himalayan *Heracleum*

The results from the combined and separate analyses have consistently revealed that the genus *Heracleum* is not monophyletic, but polyphyletic because *Heracleum* species are distributed throughout the 'Major *Heracleum clade*' and several genera (*Pastinaca*, *Malabaila*, *Semenovia*, *Zosima*, *Tordyliopsis* and *Tordylium*) are nested within *Heracleum sensu lato*. Two main lineages have been identified within Sino-

Himalayan *Heracleum* (Fig 8.1, 8.2).

***Heracleum* Group A1** (Fig. 8.1) is well supported and characterized by highly reduced calyx teeth (absent or poorly developed), broad and inconspicuous dorsal ribs of the fruit, and distinctive clavate vittae which are more or less half the length of the mericarp. We have divided this group into three sub-groups (B1, B2, B3) which correlate to geographical distribution.

Sub-group B1 is strongly supported (87% bootstrap value) and contains *H. franchetii*, *H. scabridum, H. stenopterum* and *H. forrestii* species which are exclusively from SW and NW China. Within this group the samples of *H. franchetii* and *H. scabridum* form well supported groups and appear to be good species, although the similarity of vegetative characters across this sub-group makes identification very difficult as the species boundaries are often blurred. Very few differences in sequences were seen between these and the other samples in this group, and it is notable that the *H. stenopterum* and *H. forrestii complex* species have identical or only 1-2 base pair differences between the sequences. More extensive sampling across China will be needed to resolve the taxonomic problems in this group.

Sub-group B2 is weakly supported, but forms a distinctive group which is mainly composed of species allied to *Heracleum sphondylium* from NE China including Korea and Japan, through to North America (*H. lanatum* 2). Within this sub-group only species from Korea (*H. moellendorfii*) and from Japan (*H. lanatum* 1) form a well supported group. The past identification of Japanese *Heracleum* taxa as *H. lanatum* appears dubious because not only is this taxon morphologically very similar

to *H. moellendorfii* from Korea, but also it is now seen to be genetically very close to this species with only two bp differences and rather different to the North American *H. lantatum*. NE Chinese taxa (*H. tiliifolium* and *H. moellendorfii*) and one North American taxon (*H. lanatum* 2) are not resolved in our analyses.

There is a classic distribution pattern seen in other Apiaceae genera (Wen, 1999): e.g. *Osmorrhiza* (Wen *et al*., 2001) and *Crytotaenia* (Li, 1952). *Heracleum lanatum* can be thought to represent a disjunct distribution between Asia and eastern North America, if the Japanese taxon is real *H. lanatum*. But at the moment this species can not be thought of as a disjunct species because the identification of the Japanese taxon is uncertain. Probably further work on this species near beringian area (the distribution of *H. lanatum* is previously reported) will be needed to clarify the distribution.

In the ML analysis the *H. mantegazzianum* group arises inside sub-group B2 (Fig. 8.1) and sister to the *H. moellendorfii* group. But in the BA analysis this group is isolated and sister to all other Sub-group B1 species. Sub-group B3 consists of *H. sphondylium* and *H. dissectum* as sister to all groups A1 and A2. These taxa have identical sequences even though they were collected from widely different areas. *H. sphondylium* from UK and *H. dissectum* from Central Siberia (Russia) forms well supported sub-group B3. Although *H. dissectum* is often distinguished from *H. sphondylium* on the basis of overall size and degree of leaf dissection, many people identify them on chorology alone.

The taxonomy of the *Heracleum sphondylium* complex is highly problematic with

many species described in the past based on unreliable characters of differences in leaf segmentation, lobe and tooth architecture. Recently, taxonomic treatments of European taxa now treat *H. sphondylium* as a single morphologically very variable species, and such a broad species concept has been followed by Briquet (1924), Thellung (1925-1926), and Brummitt (1972). North American *H. lanatum* has also been treated within *H. sphondylium* (as subsp. *montanum*) by Brummitt (1972). In Japan this broad species concept has also been adopted in *Flora of Japan* (Ohba, 1999), and all Japanese *Heracleum* taxa are treated under *H. sphondylium*. Our results generally support these treatments, although further study would be needed with broader sampling before sweeping changes can be made to the classification of this complex worldwide.

8.4.4 *Heracleum* Group A2, A3, *Semenovia*, *Tordyliopsis*

These are plants from the mountains of the Himalayan region. Although this group appears to be a natural assemblage of species, it is, however, poorly supported, with a bootstrap value of less than 50%. Within this larger group several sub groups can be usefully recognised.

***Heracleum* group A2** (Fig. 8.1, 8.2)

Heracleum wallichii forms a monophyletic group with its sister species *H. sublineare.* They are very closely related and sometimes difficult to separate on morphology alone. However, fruit wing characteristics, such as broadness of the marginal wing are diagnostic, with *H. wallichii* having wings definitely broader than the fruit body.

Heracleum nepalense and *H. woodii* forms a well supported group with sister to *H.*

kingdonii. Heracleum nepalense is morphologically a rather variable species, widely distributed across the Himalaya and into SW China, and is often misidentified in herbaria. *Heracleum woodii* was described by Watson (1999) in *Flora of Bhutan* and separated from *H. nepalense* using number of characters (yellowish green flowers, small number of rays, solitary subtending lateral umbel), but many of the fruit morphological characters such as wing margins and dorsal ribs characters are very similar to *H. nepalense.* In the past *H. woodii* has been treated as *H. nepalense* D. Don var. *bivittatum* Clarke, and our ITS and ITS + rps16 combined analyses also shows their close relationships. Wider sampling of *H. nepalense* would be desirable to confirm this relationship and to indicate the most appropriate rank for the Bhutanese plant. However, it is worth noting that in the present study *H. nepalense* has proven remarkably difficult to extract good quality DNA and of the many accessions sampled, only one produced usable sequences.

Heracleum hookerianum and *Tetrataenium rigens* (Wall. ex DC.) Manden. (=*H. rigens* Wall.) are restricted to the southern part of India (Nilgiri hills, Peninsular India). They are not well resolved, but cluster with the Himalayan *H. bhutanicum* and a new species of *Heracleum* from West Yunnan. They share yellow or yellowish-white flowers and terminal and lateral peduncles. Because of its flower colour and inflorescence structure, several south Indian *Heracleum* taxa (e.g. *Heracleum sprengeleanum* Wight & Arn., *H. candolleanum* (Wight & Arn.) Gamble, etc.) have in the past been included within the genus *Pastinaca* (Wight & Arnott, 1834). Our analyses confirm that at least two of these South Indian *Heracleum* taxa should be included in *Heracleum* rather than *Pastinaca*. The *Heracleum bhutanicum* group has good bootstrap support, but the relationships with other nearby species are not clear.

H. bhutanicum is endemic to Bhutan and this is low altitude, yellow flowered, hairy species, is only known from the Chukka district SW Bhutan. The new species of *Heracleum* "*H. new species"* was very recently collected by Jin-Hyub Paik from the remote mountain of Chukai in NW Yunnan, China. This is the subject of further ongoing investigation and will be describe later. Preliminary study of fruit anatomy shows that it is different from all known *Heracleum* species.

***Heracleum* group A3**

Heracleum candicans and *H. obtusifolium* with *H. pinatum* and *H. canescens* form group A3 which is moderately supported. However, within this group, it is notable that *H. candicans* and *H. obtusifolium* form a strongly supported monophyletic group in all analyses. These two species are widely distributed across Sino-Himalaya and very variable, particularly in the size and dissection of the leaves and the shape of the leaflets. The distinction between two species has long been problematic, with Cannon (1978) commenting that *H. obtusifolium* is probably only a minor variant of *H. candicans*, and suggested that further investigation is need to confirm its status. Pu & Watson (2005) treated *H. obtusifolium* as variety of *H. candicans* (*H. candicans* var. *obtusifolium* (Wallich ex DC.) F. T. Pu & M. F. Watson), and our phylogeny confirms that these two closely related taxa are indeed difficult to separate and are more appropriately treated as conspecific.

Tordyliopsis brunonis

It has been treated as a *Heracleum brunonis* by past authors (e.g, Pimenov *et al*., 2000). Our results show that this species is rather distinct from most Himalayan *Heracleum* species, but it is closely related to some other *Heracleum* species and

the separation of *Tordyliopsis* from *Heracleum* purely on our molecular evidence is not strongly supported. De Candolle (1830) described this monotypic genus on the basis of the distinctive involucre of several narrow, tomentose, mostly subentire bracts about half as long or longer than the rays, and involucel of conspicuous bracteoles similar to the bracts. In our micro-morphological examination using SEM we found that *Tordyliopsis brunonis* has unique glandular trichomes not seen in the other *Heracleum* species (See chapter 5). On the basis of combined evidence we uphold the treatment of this distinctive species in a separate monospecific genus.

Heracleum cachemiricum* and *Semenovia

Heracleum cachemiricum always appears in the present study as closely related with two species of *Semenovia* (list them). Alava (1987b) once transferred this species to *Tricholaser* Gilli, as *T. cachemericum* (C. B. Clarke) Alava on the basis of the hermaphrodite and male flowers in the same umbellule, the slightly tumescent fruit wing margins, and subequal dorsal vittae extending nearly to the fruit base. However, this classification was not adopted by recent floristic account in Himalayan regions (Mukherjee & Constance, 1993; Nasir, 1971). The present study highlights the distinctness of *H. cahemericum* from the other sampled *Heracleum* species, but further sampling of *Semenovia* in Central Asia will be required to clarify its taxonomic position.

Semenovia is mostly a Central Asiatic genus (SW, S, Central Asia) comprising around 18 species, sometimes included within and sometimes separated from *Heracleum* (Pu & Watson, 2005; Watson, 1999). This genus circumscribed by linear dorsal vittae which run the full length of the mericarp, fruit wing margins that are not

inflated, and an unsclerified inner mesocarp layer (Alava, 1987a; Pu & Watson, 2005). However, the delimitation of this genus has been problematic. In their treatment of the Afghanistan representatives, the *S. lasiocarpa* complex, Rechinger & Rieldl (1963) stated that “the taxon is a very polymorphic one so the treatment is considered provisional.” In *Flora Iranica* Alava (1987a) also suggested the necessity of a more compressive survey of the genus because many of the these species are known only from scanty and fragmentary type collections many of which lack characters of diagnostic value (e.g. mature fruit, flowers , basal leaves). In our analyses, two species of *Semenovia* (*S. transiliensis*, *S. dasycarpa*) always appears closely related with *Heracleum* Group A2 and A3. Results from a very recent phylogenetic study of Iranian Apiaceae using ITS sequences (Yousef *et al.*, In press) also supported our results. In this study, two different species of *Semenovia* (*S. dichotoma* and *S. fragioides*) are also nested inside their ‘*Heracleum* clade’ and are placed close to the *H. candicans* group. Clearly, before any taxonomic changes can be proposed, further work on this enigmatic genus is needed, with more extensive sampling beyond the four species included in these two studies.

Apart from two *Semenovia* species, many species within our *Heracleum* Group A2 and A3 correspond to Mandenova’s (1959, 1982) genus *Tetrataenium*. This generic circumscription is further supported by fruit morphological characters: well-developed calyx teeth; prominent and narrowly carinate dorsal and intermediate fruit ribs; and elongate, truly claviform and septate dorsal vittae. However, adopting a broader circumscription of this genus to cover all elements in our *Heracleum* Group (A2, A3) would be far reaching and premature. Before this, further studies on the morphological and anatomical characters of these species are need together with

more exhaustive sampling for molecular analysis, especially of *Semenovia*.

8.4.5 Relationships between *Heracleum* and its sister genera

***Pastinaca-Malabaila* group** (Fig. 8.1, 8.2) includes *Pastinaca* (cultivated worldwide) and *Malabaila* (Mediterranean) in a group with a strong support (bootstrap 99%) and characterized by yellow petals, and simply pinnate leaves. It is interesting to note the placement of this group close to the core *Heracleum* group (*Heracleum* Group A1), a result also supported by other recent studies (Yousef *et al.,* In Press; Logacheva *et al*., In Press). Calestani's (1905) rather radical classification of these genera included *Malabaila*, *Heracleum*, *Zosima*, *Lophotaenia*, *Ainsworthia*, *Wendiana*, and *Tordylium* within *Pastinaca*. Later studies refuted this classification, and Menemen & Jury (2001a, b, c) suggested that these genera could be clearly separated from each other using characteristics of macro-morphology, micromorpholgy, anatomy, and palynology. Our molecular analyses also support treating these genera as distinct, but in our results the taxonomic relationships between species of *Pastinaca* and *Malabaila* are still ambiguous. In our results the placement of *Malabaila* is still unresolved and closely related to *Pastinaca* s. str., but the remaining species are dissimilar in fruit characters (See chapter 6, 7). Pimenov & Ostroumova (1994) proposed remove these remaining species and treating them in the neglected genus *Leiootulus* Ehrenb. as the difference of carpological structure of its type species, *Malabaila graveolens* (*P. clausii*). However, later detailed morphological analyses by Menemen &Jury (2001a) showed no nomenclatural changes were necessary.

The *Zosima* group

Group D is strongly supported with a bootstrap value of 99% (Fig.8.1, 8.2). This distinctive genus is characterised by its large vittae, which completely fill the space between the dorsal ribs, the linear leaf segments (Menemen & Jury, 2001c), and the development of distinctive parenchyma cells in the proximal regions (See chapter 6). It also has a distinctive composition of flavonols and flavones (Menemen *et al*., 1998), and in all our analysis it clearly forms a monophyletic group. The relationship of this group to the other groups is less clear as it forms a polytomy almost at the base of the ITS phylogeny and appears in a polytomy rather higher up the tree in the ITS and rps16 intron combined analysis. Further sampling with *Zosima* and using other gene sequences is needed resolve these issues.

8.4.6 Taxonomic placement of some enigmatic taxa

The taxonomic position of ***Heracleum millefolium*** has long been problematic. Wang & Shan (1993) recognised the uniqueness of this species and placed it in a new Section within *Heracleum* (Sect. Millefolia Wang & Shan) based on the vittae shape, size and basal leaf arrangement (3-4 pinnate). In the Flora of Bhutan (Watson, 1999) followed Central Asian treatments and recognised this within *Semenovia* (*S. millefolia*) based on the dwarf perennial habit with a tough fibrous stem base, mostly basal pinnate leaves and fruit with dense white hairs. In the Flora of China (Pu & Watson, 2005) this species was placed again treated within the genus *Heracleum* on account of the radiate outer flowers and clavate vittate. The results of the present study show that the taxonomic position of *H. millefolium* is still uncertain, but at least its separation from all *Heracleum* species sampled (and the other allied genera sampled including *Semenovia*) is supported. Even though the

taxonomic position of *Heracleum millefolium* is not strongly resolved, other morphological characters (such as habit, basal leaf arrangement, trichome features of fruit and fruit anatomy) strongly support its distinctiveness. Further sampling of *Semenovia* and related genera will help to confirm its position as at the moment it does not sit well in either *Heracleum* nor *Semenovia*. The results of the present study suggest that the description of a new genus for this enigmatic species may well be appropriate.

The taxonomic position of ***Tordylium*** has been contentious because previous classifications were wholly based on suites of morphological characters with no single character being diagnositic. Al-Eisawi & Jury (1988) revised the genus *Tordylium* and the four related genera *Ainsworthia* Boss., *Tordylium* L., *Synelcosciandium* Boiss., and *Mandenovia* Alava. In this revision *Ainsworthia* and *Synelcosciadium* were treated within *Tordylium*, and *Mandenovia* as a monotypic genus. The genus *Tordylium* was divided into subgenus *Tordylium* (including *Synelcosciadium*) and subgenus *Ainsworthia* (Boiss.) Drude. In our phylogenetic analysis we included two species of *Tordylium* (*T. apulum* and *T. elegans*), which do not cluster together and are widely placed in all our trees. Al-Eisawi & Jury (1988) placed both of these species in subgenus Ainsworthia, but *T. apulum* within section *Condylocarpus* (Hoffm.) DC. and *T. elegans* within section *Hasselquistia* (L.) Boiss. In other recent studies *T. aegyptiacum* var. *palaestinum* is also oddly placed (Yousef *et al.* In press), and *T. maximum* (the type species of the genus) is placed sister to the *Pastinaca* and *Heracleum* group (Locheva *et al*. In press). Our results and these recent studies indicate that a detailed molecular study of *Tordylium sensu lato* could be very useful in helping to resolve these long-standing problems.

8.4.7 Sectional classification of Chinese *Heracleum*

Although, *Heracleum* group A1 is well supported and comprise of three subgroups, is mainly consist of species of Section *Heracleum*, with the exception of *Heracleum mantegazzianum* that has been placed either in Section *Pubesentia* (Mandenova, 1950, 1962, 1982, and 1987) or Subsection *Pubesentia* (Satzyperova, 1984). According to Mandenova, Sections *Heracleum* and *Pubesentia* differ only in dorsal mericarp vittae being narrow, extending near the mericarp base and there sharply enlarged; commissural vittae are similar to those of Section *Heracleum*. Satszyperova (1984) showed also some differences in pollen structure between the two higher taxa. The results of the present study show that *H. mantegazzianum* should be included in Section *Heracleum* and is closely related to *H. moellendorfii* and *H. lanatum* which are typical species of Section *Heracleum*. This relationship is also supported by the other characters such as fruit surface (see chapter 7). It is likely that the morphological differences used to characterise Section *Pubesentia* are not reliable and that this Section should be included within Section *Heracleum*.

Group A3 it is represented mostly by members of the Section *Villosa* (Mandenova, 1950, 1962, 1982, and 1987; He *et al*., 1998; Zhao *et al*., 2004) or Section *Tetrataenium* (De Candolle, 1830). *Heracleum pinnatum* has not been considered within any sectional classification, but has been attributed to the genus *Tetrataenium* (Mandenova, 1982). The difference between Sections *Heracleum* and *Villosa*, according to Mandenova, are in the features of vittae. In Section *Heracleum* vittae in the dorsal valleculae are clearly clavate, and on the commissural side they are extended to a half of fruit length as measured from the stylopodium to the base of

mericarp. Section *Villosa* have, in contrast, large dorsal vittae of approximately equal width (non-clavate), and commissural vittae which extend from 1/2 to 1/3 of the mericarp length. For the Chinese *Heracleum* (*H. candicans*, *H. obtusifolium*) Wang & Shan (1993) followed Mandenova's concept of Section *Villosa* in general, but He *et al*. (1998) and Zhao *et al*. (2004) added more useful characters such as densley white tomentose at abaxial side ,pollen shape and petiole anatomy to characterise the Section. Our molecular results somewhat support the separation of Section *Villosa* from other *Heracleum* species but it is very weakly supported and fruit anatomical characters (such as distinct dorsal ribs, narrowly distributed, vittae linear, see Chapter 6) are rather continuous with Group A2. Furthermore, because Mandenova' section *Villosa* was mostly based on W Asian species, the characters for section *Villosa* might be not compatible to W Chinese species. A recent study of West Asian *Heracleum* (Rogacheva *et al*., 2007) has revealed that the species previously included in this Section were no longer concordance with section *Villosa*, but should be included in Section *Heracleum*. Clearly the taxonomy is still in a state of flux, and a great deal of further work is required on the Chinese species of Section *Villosa*. However, the molecular results in the present study show the utility of these techniques in potentially resolving these problems.

8.5 Conclusion

In order to evaluate the monophyly of Sino-Himalayan *Heracleum*, and clarify the species relationships within this group, additional sequences of ITS, rps16 intron and psbA-trnH spacer of *Heracleum* and related genera were analysed. Both ITS and rps16 intron regions show a good resolution of phylogenetic relationships down to species level. The comparative analysis confirmed the monophyly and refined the circumscription of '*Heracleum* clade' sensu Downie. Within this clade several major lineages are defined: *Heracleum*, *Malabaila-Pastinaca*, *Zosima*, *Semenovia*, *Tordylium*, and *Tordyliopsis*. The Sino-Himalayan *Heracleum* lineage is seen to comprise two distinct sub-lineages (*Heracleum* Groups A1, A2, and A3) including monotypic genus *Tordyliopsis* and Central Asiatic genus *Semenovia*. *Heracleum* is thus not monophyletic. Generic segregates of *Heracleum* proposed in past classifications, such as *Tetrataenium,* can be correlated with Heracleum Group A2 and A3. These phylogenies also helped resolve the taxonomic placement of some enigmatic taxa such as *Heracleum millefolium* and *Tordylium.* Section *Heracleum* is well supported, but Sections *Villosa* and *Pubesentia* do not correlate well with the present study.

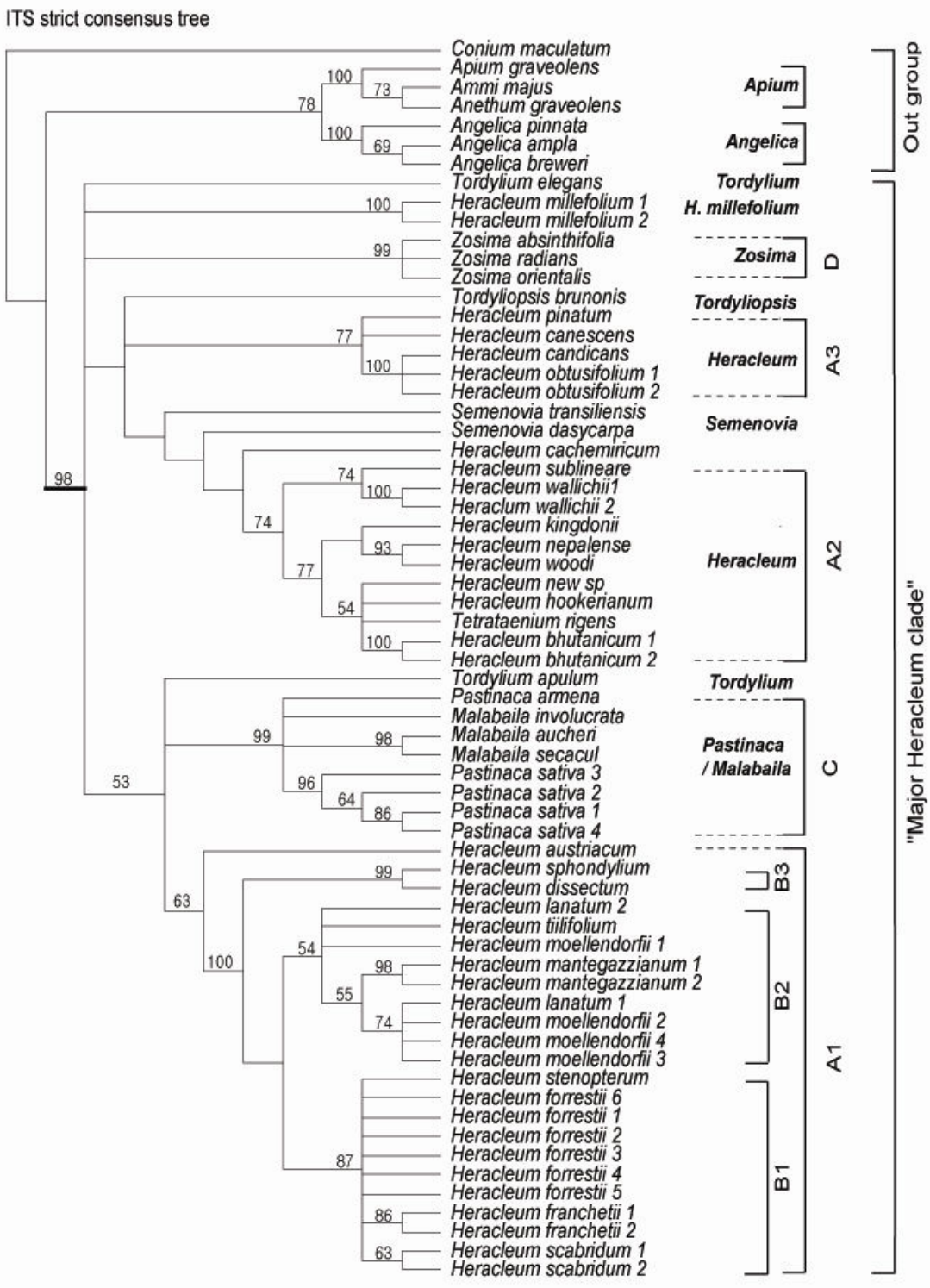


Figure 8.1 Strict consensus tree of 128 most parsimonious trees, each 584-steps long trees derived from equally weighted maximum parsimony analysis of 65 nuclear rDNA ITS sequences from Sino-Himalayan Heracleum and related genera with outgroups (CI excluding uninformative characters = 0.376; RI = 0.748). Bootstrap values are indicated along respective nodes; only those >50% are indicated. Brackets indicate clade descriptors discussed in the text.

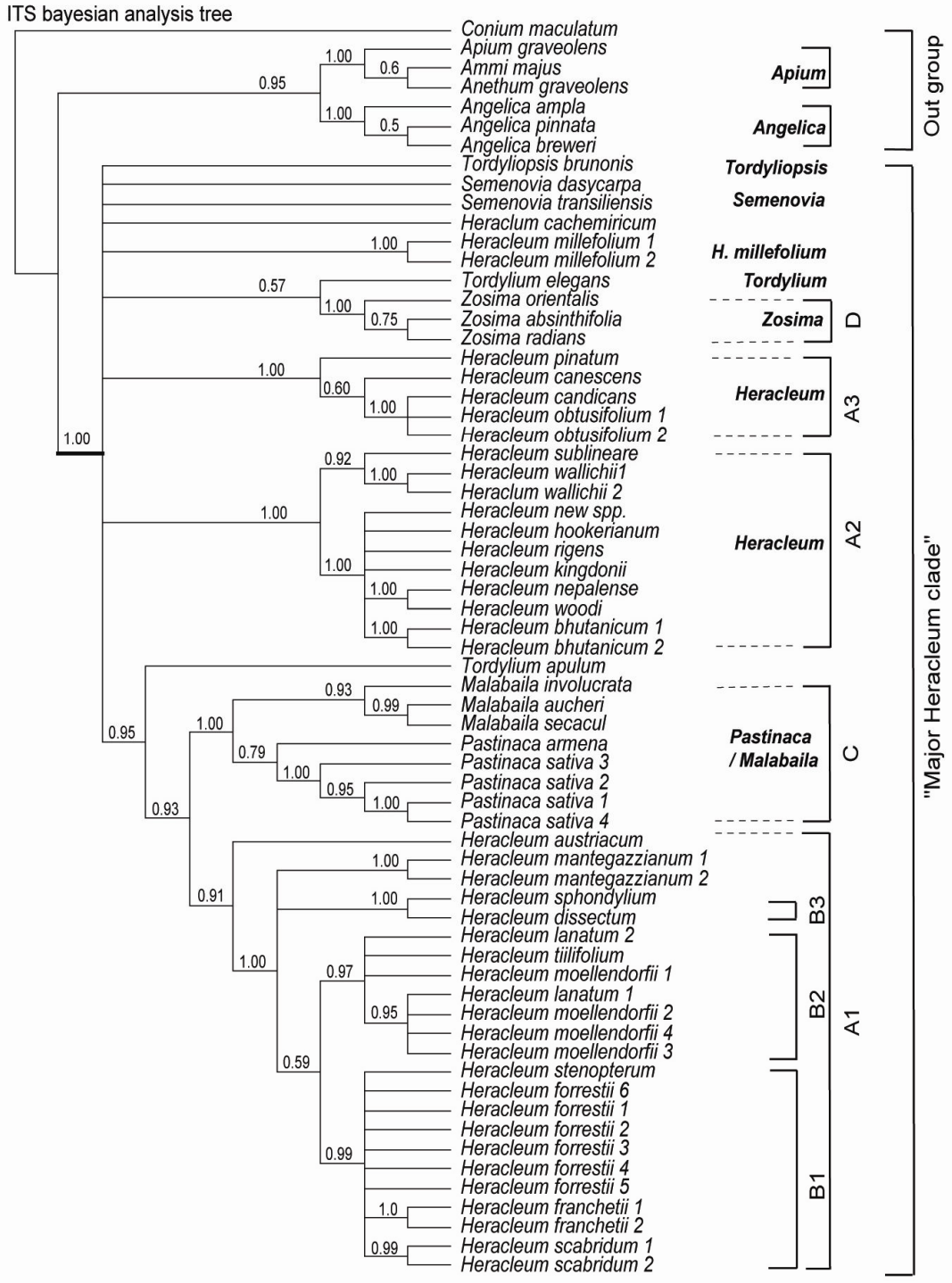


Figure 8.2 Bayesian majority rule consensus tree obtained from 65 nuclear rDNA ITS sequences from Sino-Himalayan Heracleum and related genera with the inclusion of gap characters. Numbers above braches are estimated posterior probability values.

Figure 8.3 Tree one of the 128 most parsimonious trees based on ITS sequences with a heuristic search using unweighted maximum parsimony analysis. (Tree length =584 Steps; CI = 0.611/0.521* ; RI = 0.973; RC =0.484)

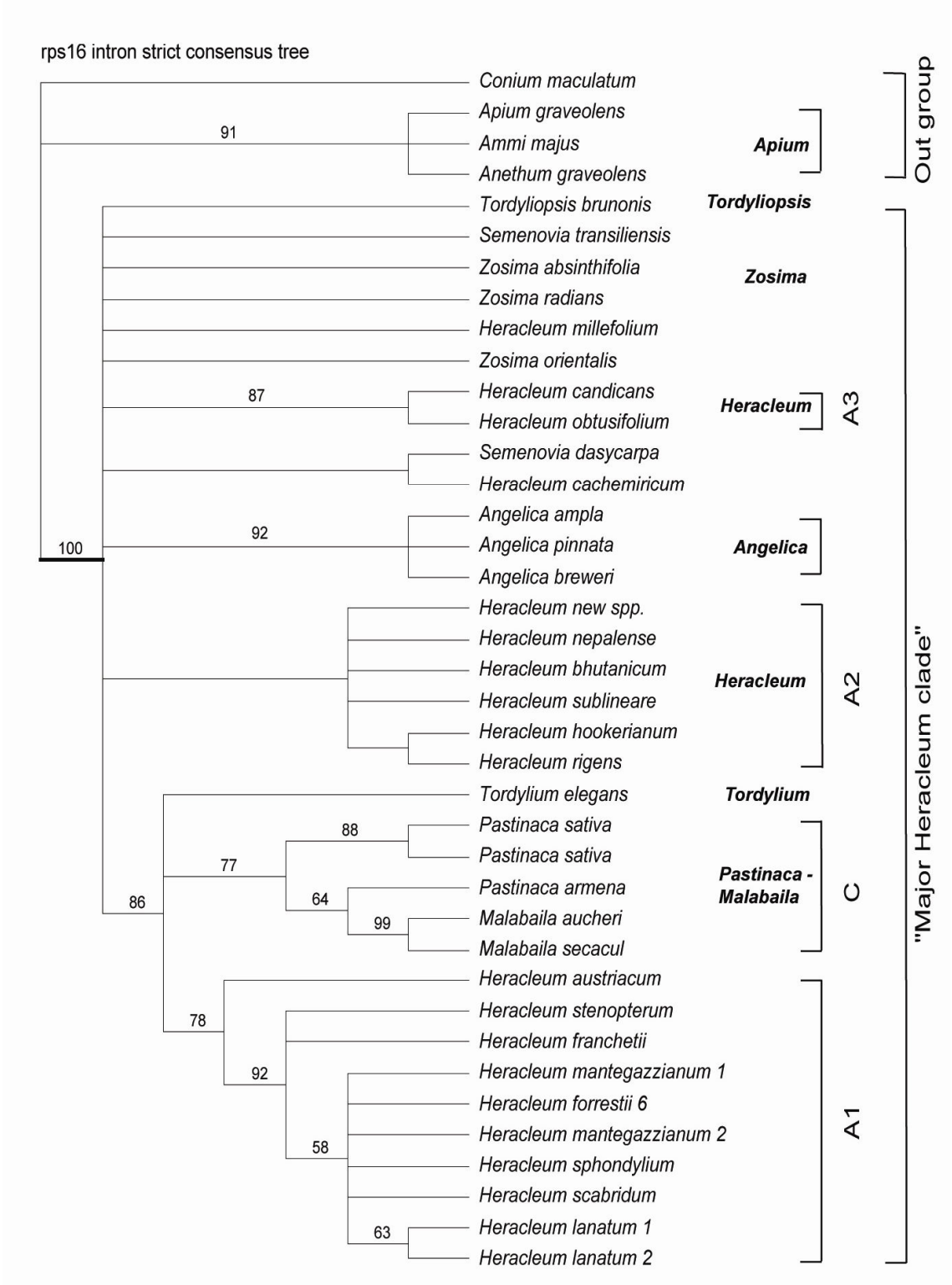


Figure 8.4 Strict consensus of 6 most parsimonious 151 steps long trees derived from equally weighted maximum parsimony analysis of aligned rps16 intron sequences from 39 accessions of Heracleum and related genera (CI = 0.467, excluding uninformative characters; RI = 0.851). Numbers on branches represent bootstrap percentage estimates from 10,000 replicate analyses. Brackets indicate clade descriptors discussed in the text.

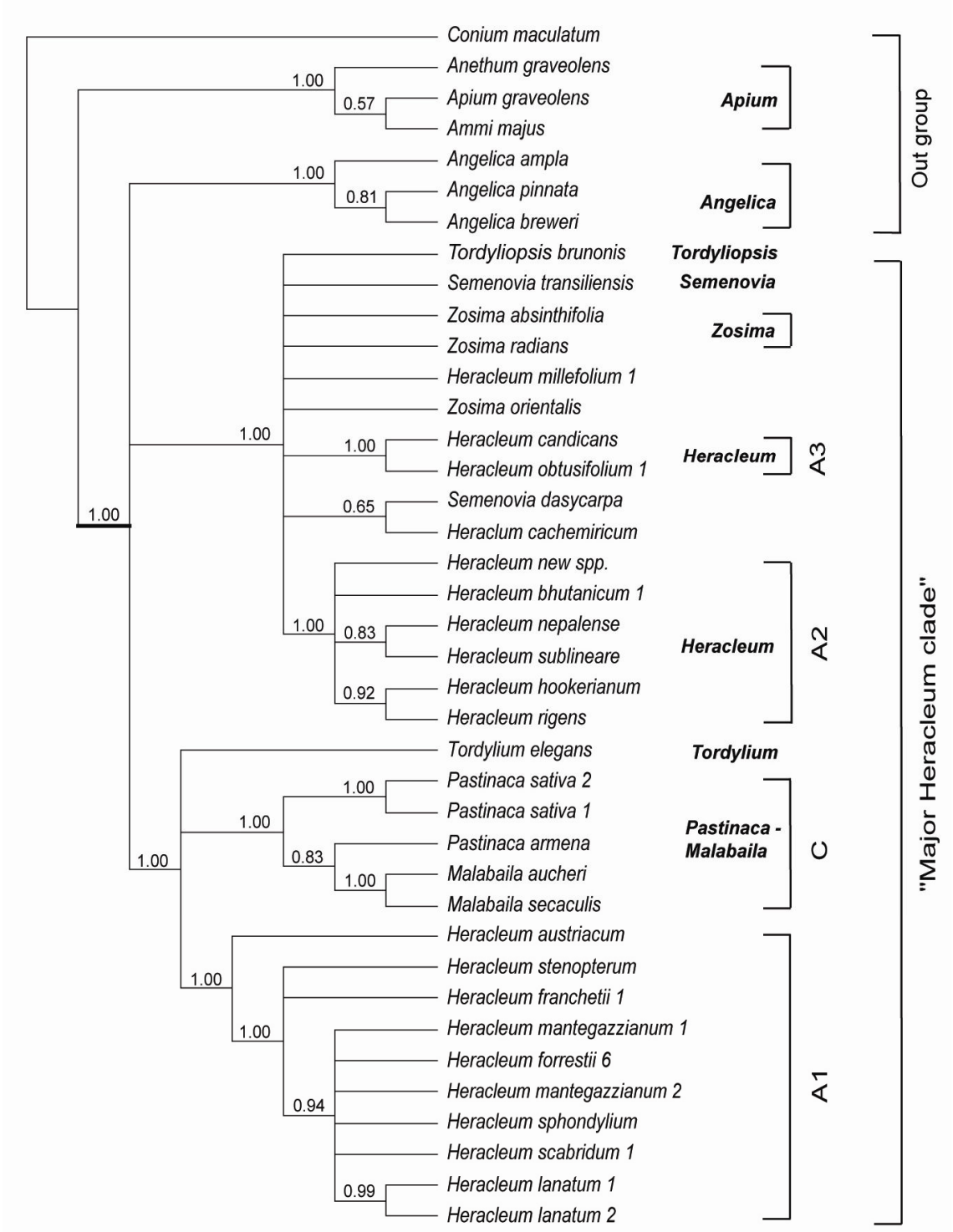


Figure 8.5 Tree produced from the Bayesian analysis of cpDNA rps16 intron sequences from 39 accessions of Heracleum and related genera. Numbers on branches are posterior-probability scores. Names of the major groups are provided next to brackets.

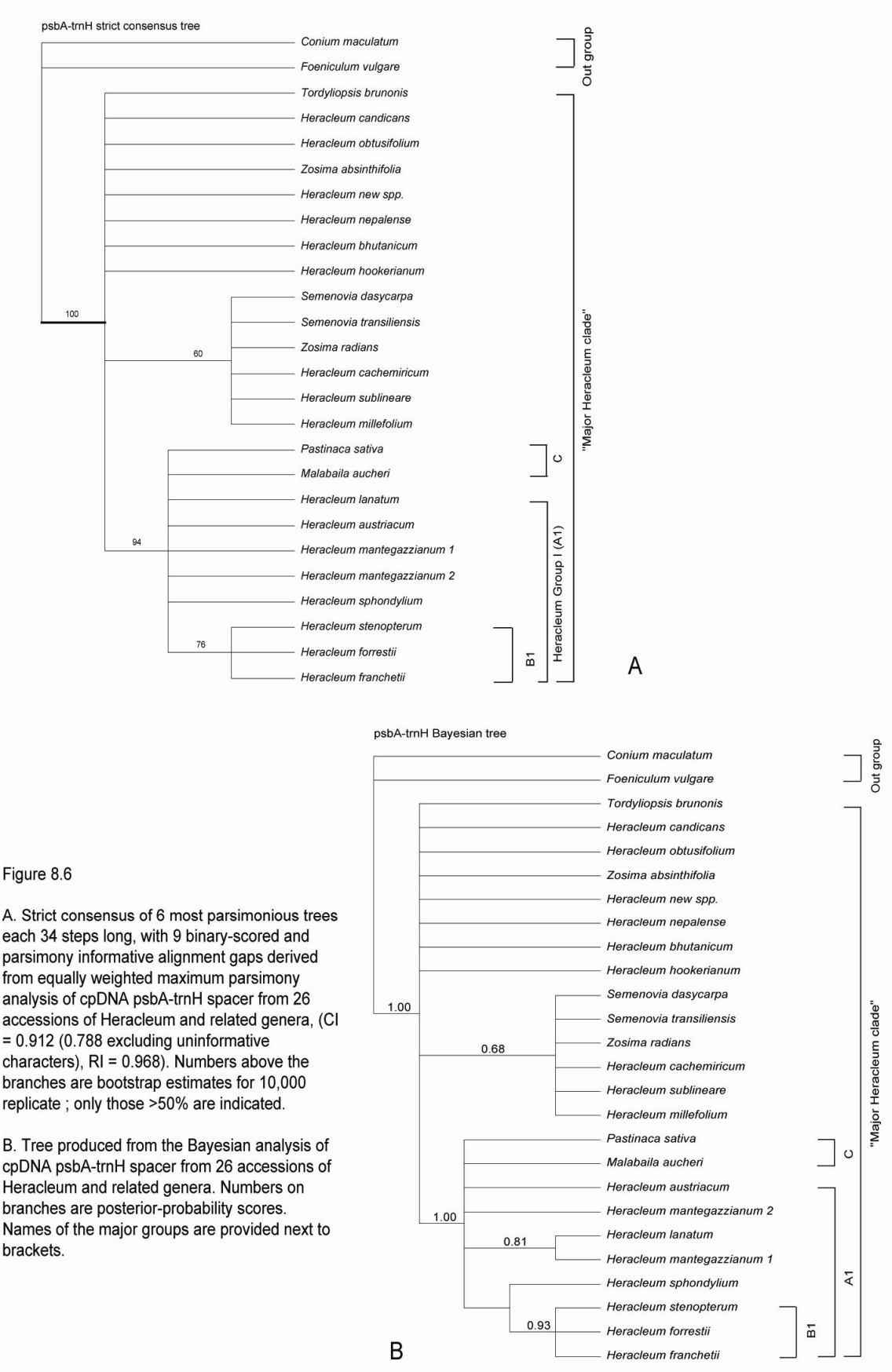


Figure 8.6

A. Strict consensus of 6 most parsimonious trees each 34 steps long, with 9 binary-scored and parsimony informative alignment gaps derived from equally weighted maximum parsimony analysis of cpDNA psbA-trnH spacer from 26 accessions of Heracleum and related genera, (CI = 0.912 (0.788 excluding uninformative characters), RI = 0.968). Numbers above the branches are bootstrap estimates for 10,000 replicate ; only those >50% are indicated.

B. Tree produced from the Bayesian analysis of cpDNA psbA-trnH spacer from 26 accessions of Heracleum and related genera. Numbers on branches are posterior-probability scores. Names of the major groups are provided next to brackets.

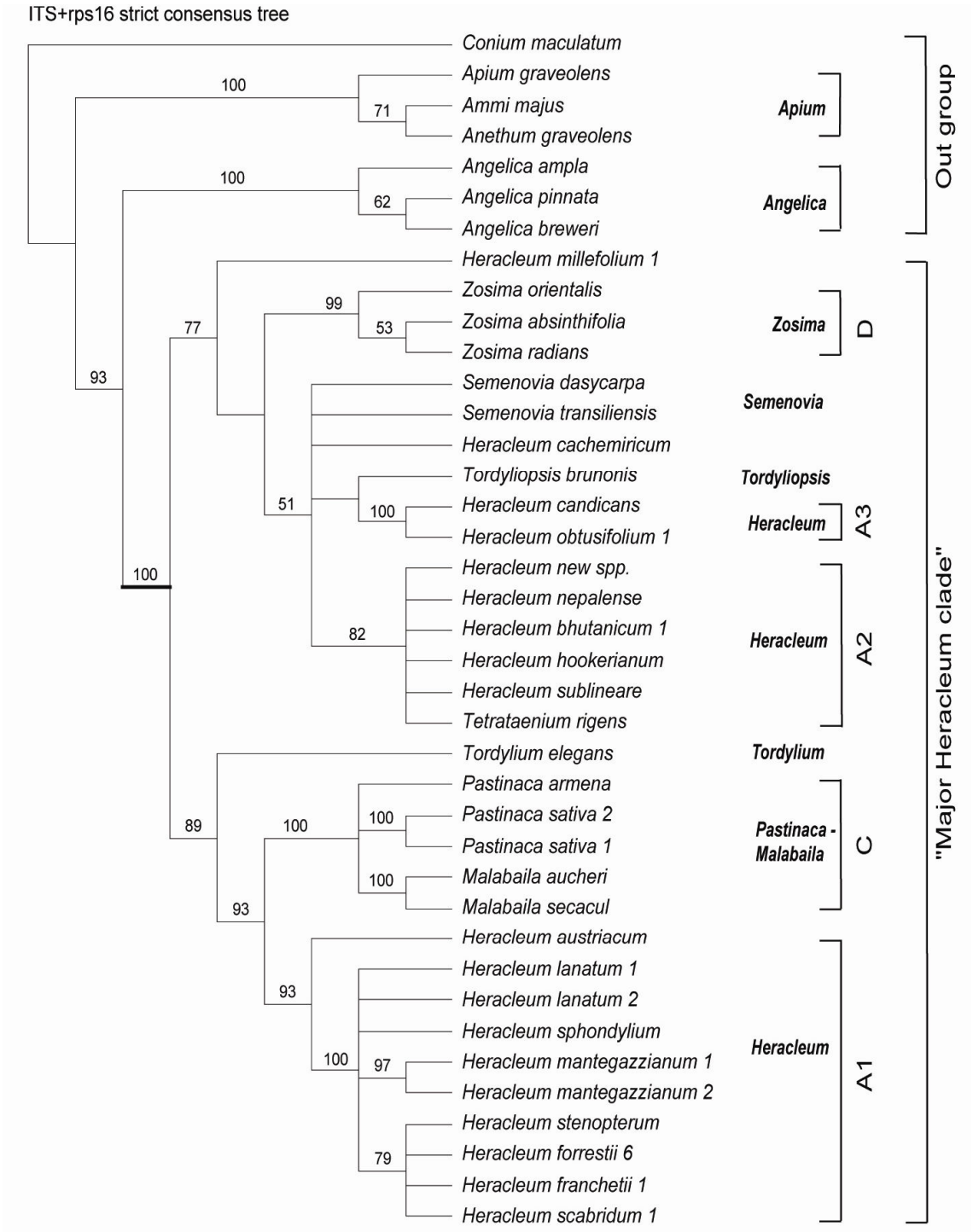


Figure 8.7 Strict consensus tree of 343 most parsimonious trees, each 667-steps long trees derived from equally weighted maximum parsimony analysis from the combined ITS and rps16 intron sequences of 39 accessions from Sino-Himalayan Heracleum and related genera. CI = 0.663 (0.484 excluding uninformative characters), RI = 0.748. Bootstrap values are indicated along respective nodes; only those >50% are indicated.

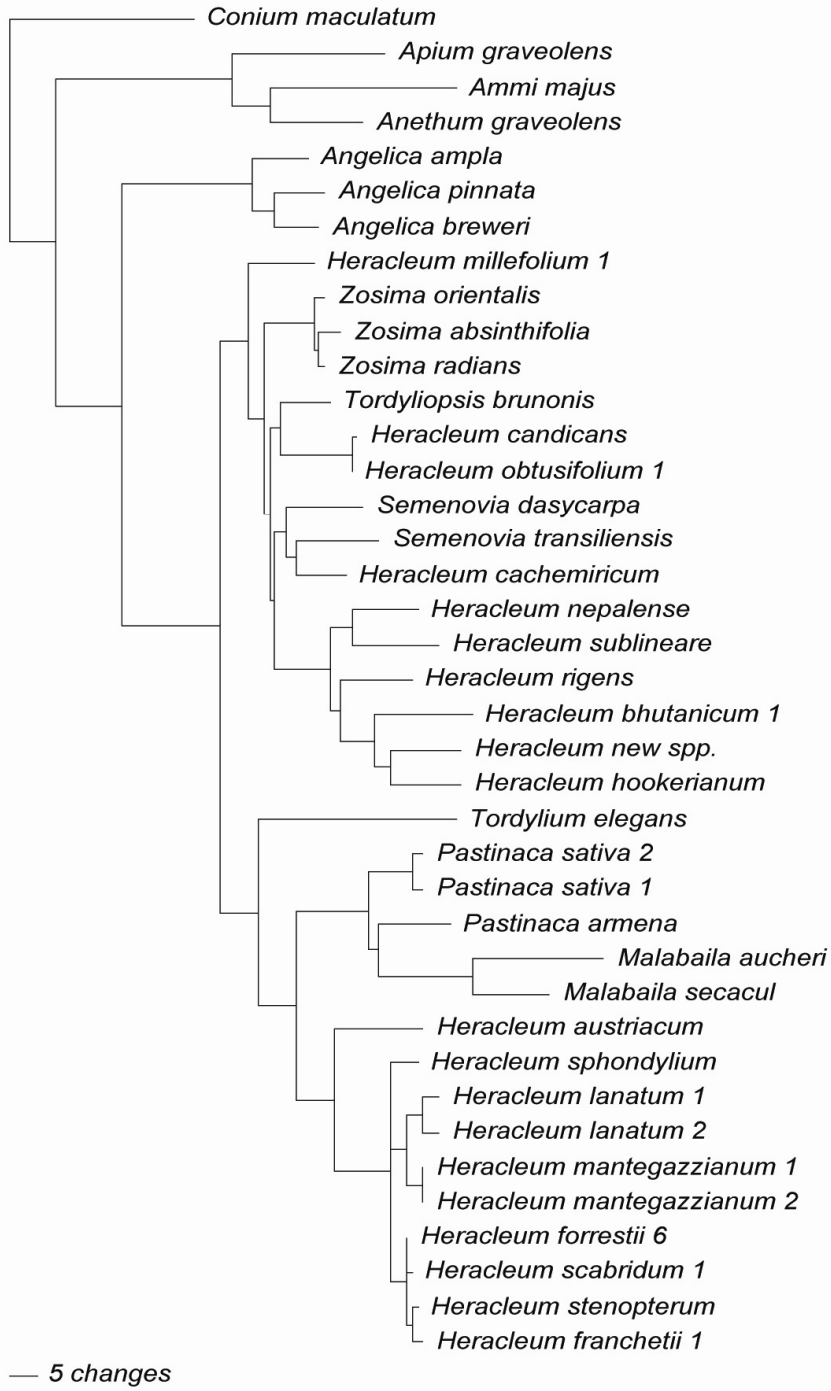


Figure 8.8 Tree one of the 343 most parsimonious trees based on combined ITS and rps16 intron sequences with a heuristic search using unweighted maximum parsimony analysis. (Tree length =667 Steps; CI = 0.663/0.484* ; RI = 0.764; RC =0.506).

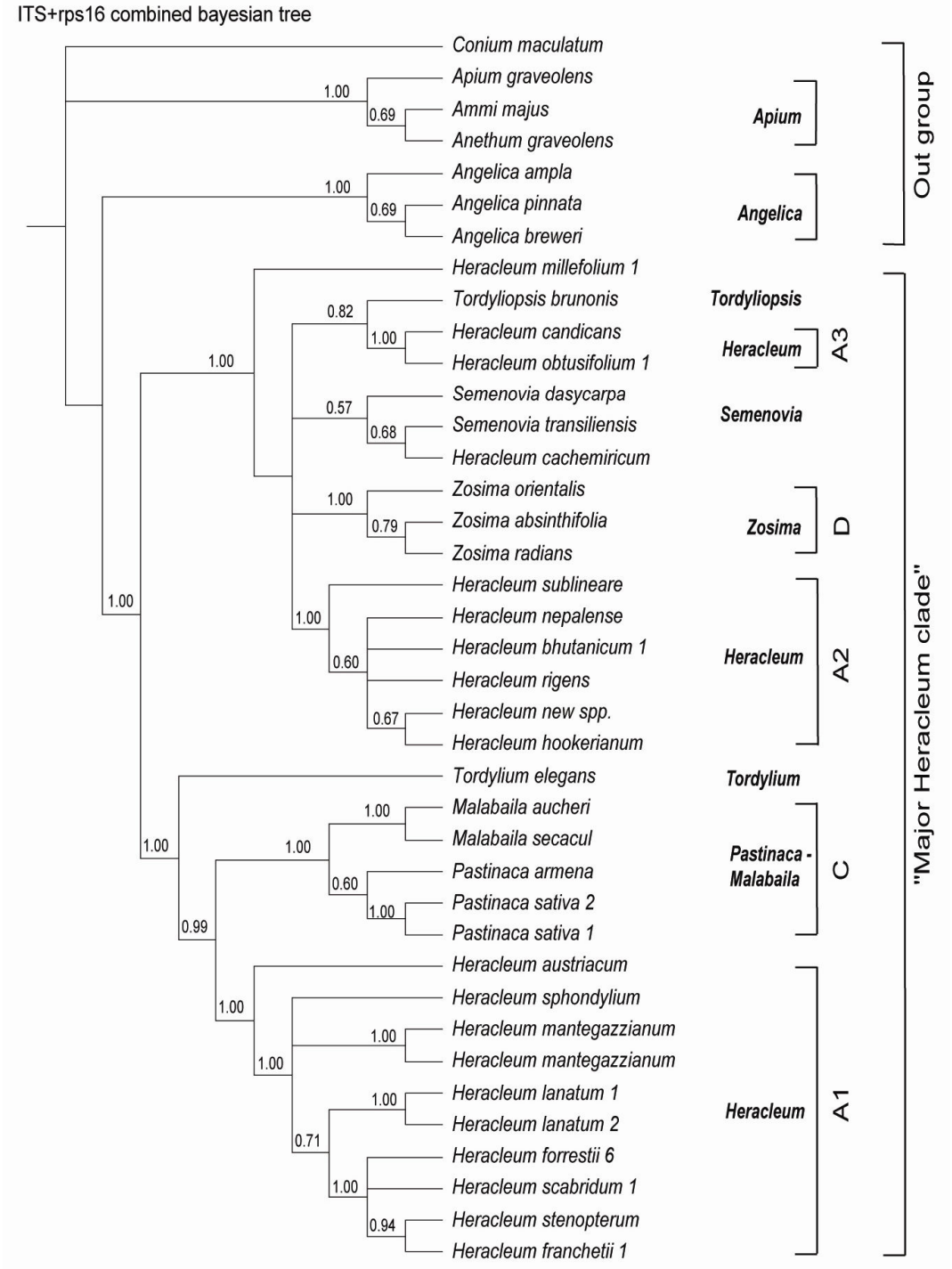


Figure 8.9 Bayesian majority rule consensus tree obtained from 39 accessions of the combined ITS and rps16 intron sequences fom Sino-Himalayan Heracleum and related genera with the inclusion of gap characters. Numbers above braches are estimated posterior probability values.

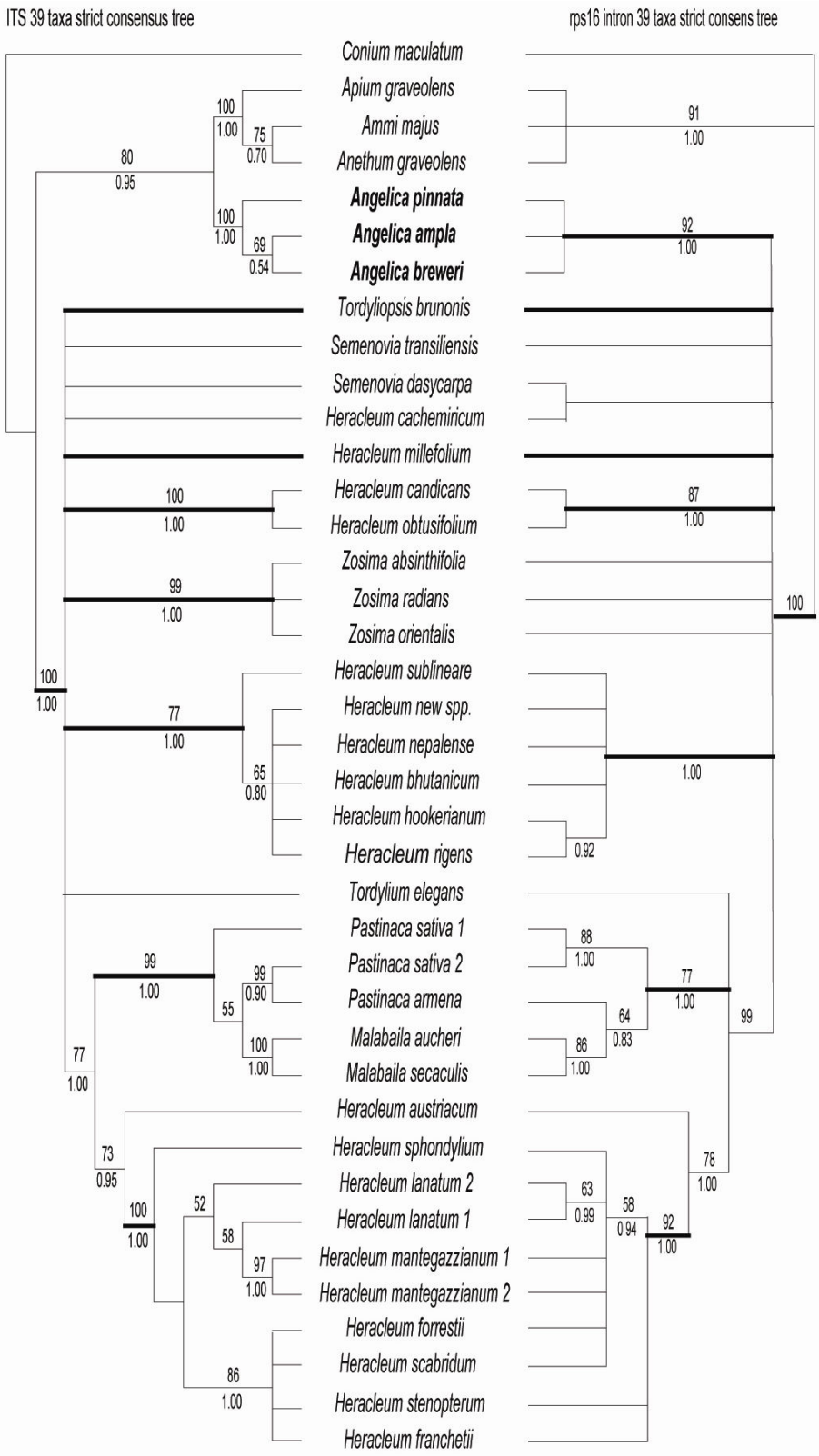


Figure 8.10 Comparison of 39 taxa of ITS strict consensus tree and rps16 intron strict consensus tree.

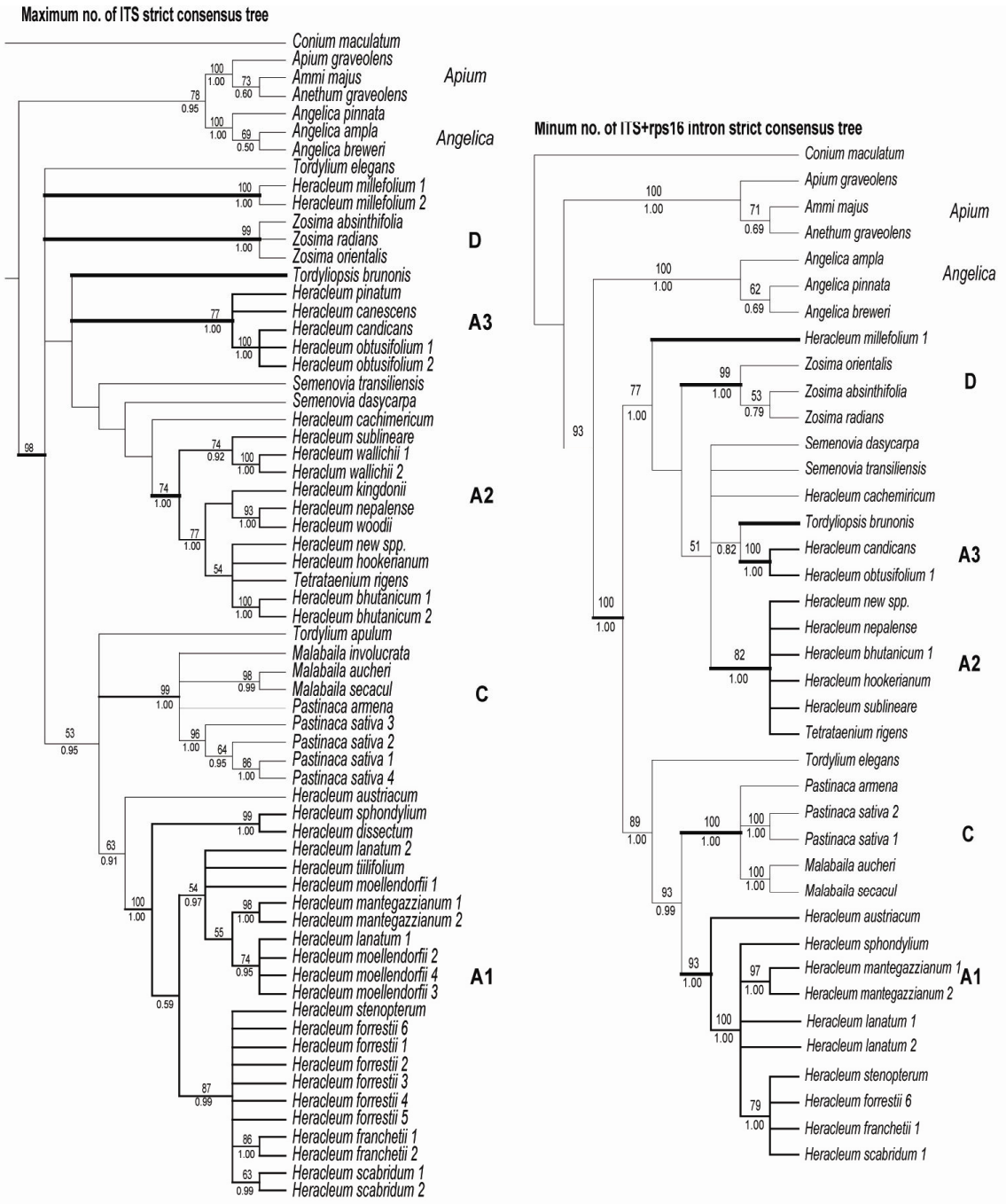


Figure 11. Comparison of Maximum number (65 taxa) of ITS strict consensus tree and minimum number (39) of ITS+rps16 intron strict consensus tree.

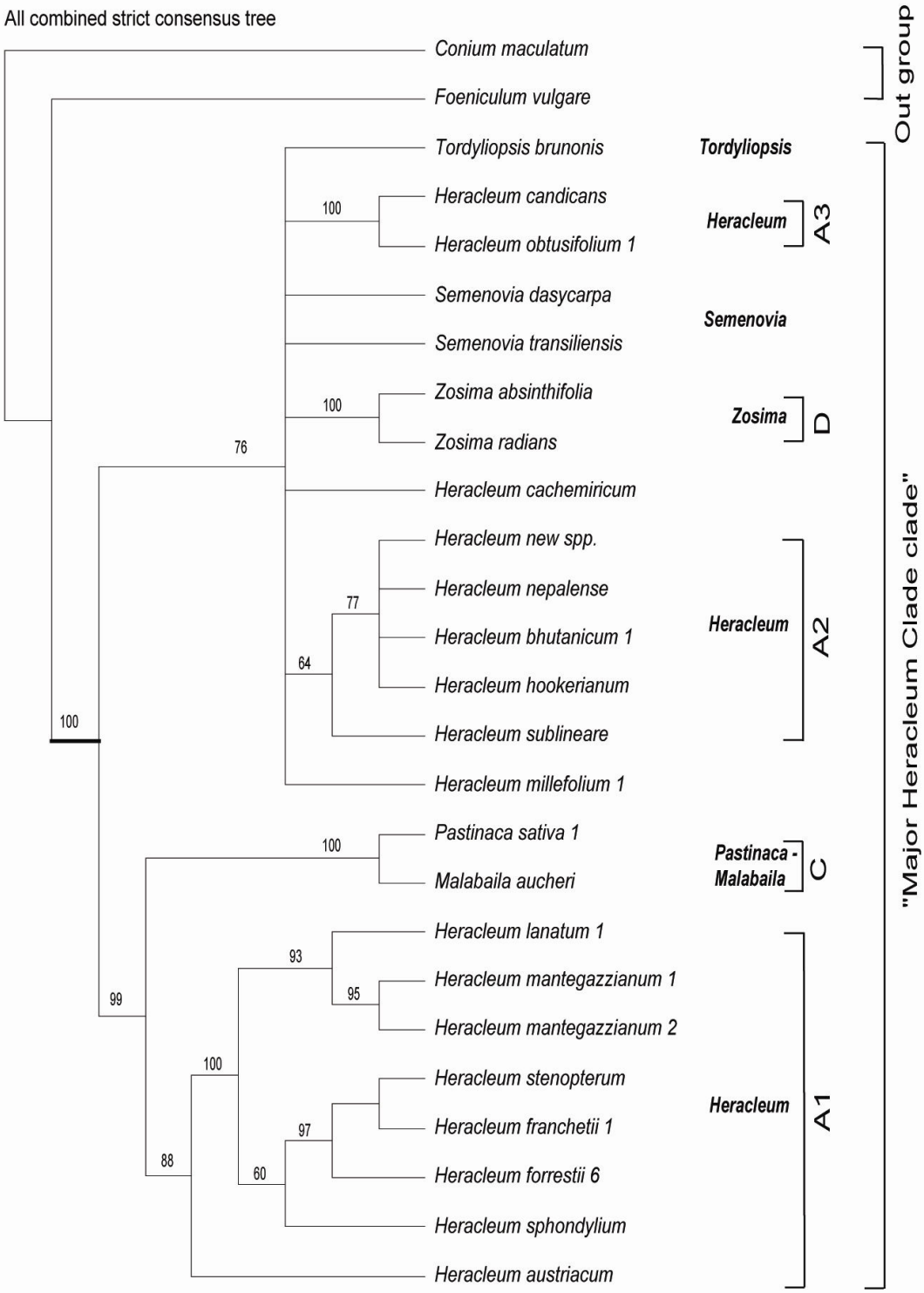


Figure 8.12 All combined (ITS, rps16 intron, psbA-trnH spacer) strict consensus tree of 33 most parsimonious trees each 492 steps long, with 9 binary-scored and parsimony informative alignment gaps from 26 accessions of Heracleum and related genera, (CI = 0.736 (0.503 excluding uninformative characters), RI = 0.766). Numbers above the branches are bootstrap estimates for 10,000 replicate ; only those >50% are indicated.

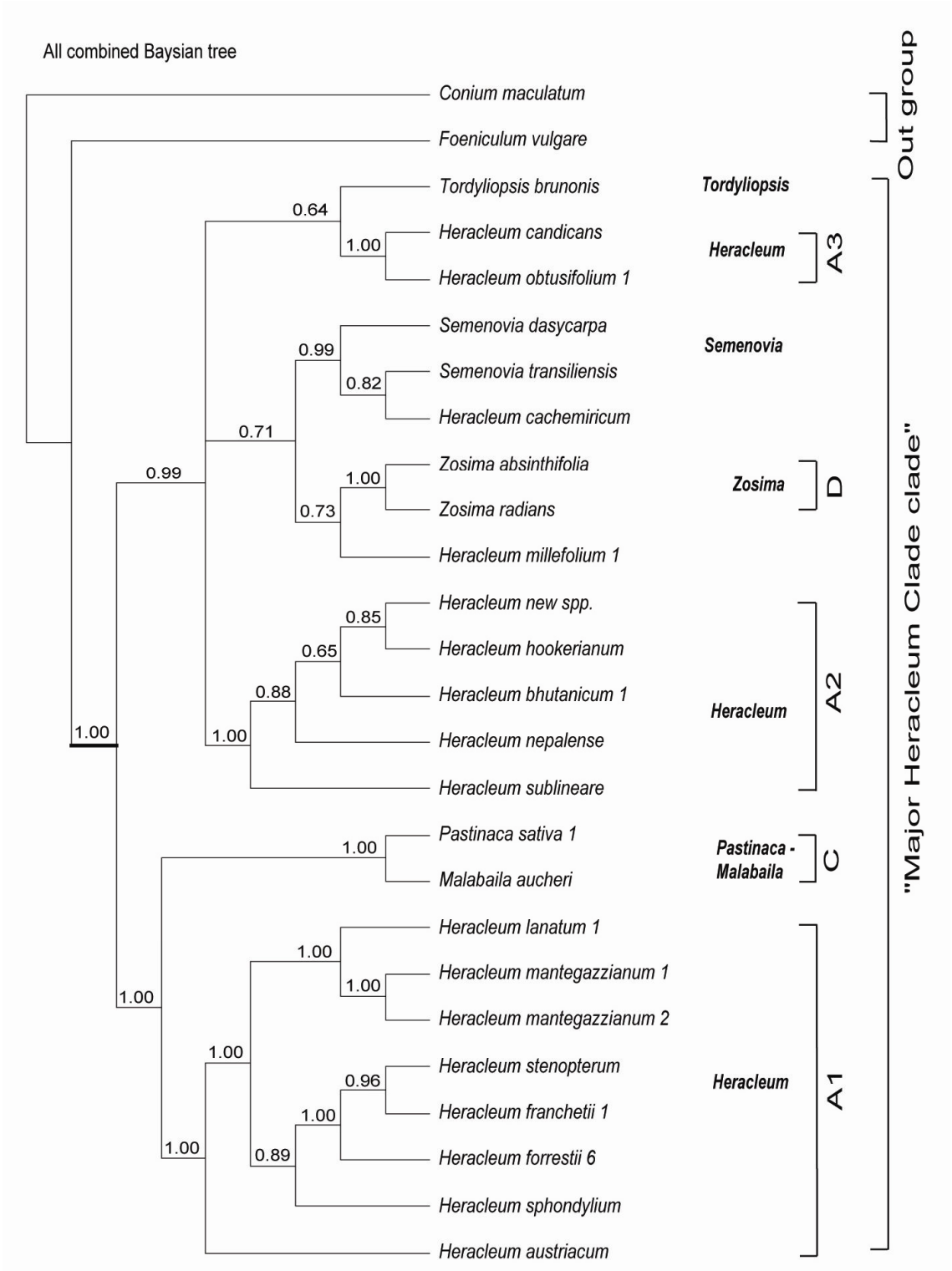


Figure 8.13 Bayesian majority rule consensus tree from all combined (ITS, rps16 intron, psbA-trnH spacer) sequences obtained from 26 accessions of Sino-Himalayan Heracleum and related genera with the inclusion of 9 gap characters. Numbers above braches are estimated posterior probability values.

Chapter 9: Conclusion

This chapter presents a synthesis of the results of this thesis, drawing together the findings of morphological and molecular investigations.

Problems of morphological complexity and blurred generic boundaries in the Sino-Himalayan region highlight the importance of *Heracleum* taxa from this area in the broader understanding of the genus and its allies. Knowledge of the Sino-Himalayan species is thus crucial for a world understanding for *Heracleum* as it is here that the problems of generic delimitation are most acute.

An examination of the literature and the specimens cited revealed that previous classifications primarily used the following macro-morphological characters to delimit taxa:

- Stem size (stout/slender)
- Cauline leaf sheath extent
- Calyx teeth shape
- Fruit wing development (fruit wing vs body ratio), and
- Dorsal and lateral rib distribution.

As explained earlier in Chapter 2, these characters which have been traditionally successfully used to delimit *Heracleum* taxa in Europe, SW Asia and North America are problematic when applied to taxa in the Sino-Himalayan region. Therefore, macro-morphology does not provide many good taxonomically informative characters with which to define ‘natural’ groups across the entire genus. The observations from the current study agree with those of Menemen &

Jury (2001a) who demonstrated that several of these 'traditional' characters, such as basal leaf shape, show considerable variation within a single species and should be used with care when delimiting taxa. In an attempt to discover further useful characters for taxon delimitation in the Sino-Himalayan *Heracelum*, the present study looked in depth at both macro- and micro-morphology.

9.1 Potentially useful sources of taxonomic data

Leaf micromorphology (Chapter 5)

A pilot study of leaf micro-morphology in Sino-Himalayan *Heracleum* (Chapter 5) was conducted in parallel with a molecular (DNA) phylogeny (Chapter 8). Several epidermal characters (e.g. trichome type, epidermal cell shape, and stomatal types) were seen to be useful in differentiating some species. In particular, *Tordyliopsis brunonis* has a unique trichome structure with apical glands. *Heracleum obtusifolium* and *H. candicans* have long, ribbon-shaped trichomes that give a lanate texture to the abaxial surface of the leaves, and *Heracleum franchetii* was the only species observed that had tetracytic stomata. However, although these characters appeared to be useful in delimiting some species or species pairs, it did not reflect any broader phylogenetic patterns as inferred from the molecular analysis. For this reason, this pilot study was discontinued in favour of a more extensive fruit morphological investigation.

Fruit anatomy (Chapter 6)

Fruit anatomy has been shown to furnish taxonomically informative characters in many genera of Apiaceae, and so a pilot study on the fruit was also being investigated at the same time as the initial study on leaf morphology. Unlike the

preliminarily results from the leaf and general morphology, those from fruit anatomy revealed more taxonomically useful characters which showed potentially good concordance with the groups revealed in the molecular phylogenies. Thus the fruit anatomy investigation was broadened and sampling intensified in order to gain a more complete dataset. The results of the final fruit anatomical study showed that allied genera and groups within *Heracleum sensu lato* are well supported on the basis of characters such as:

Heracleum sensu lato

Group 1 (Core *Heracleum* group)

- Proximal part features (thick, short), with short seed cavity.
- Mixed hypendorcarp composition (horizontal fibres and vertical fibres).
- Obsolete and widely distributed dorsal and lateral ribs
- Fruit wing's narrower than fruit body

Group 2 (*Tetrataenium* group)

- Proximal part features (thin, long) with relatively long seed cavity.
- Hypendorcarp composition (vertical fibres dominant).
- Prominent and closely distributed dorsal and lateral ribs
- Fruit wing's equal or wider than fruit body

Pastinaca

- Mixed hypendorcarp composition (horizontal fibres and vertical fibres).
- *Pastinaca* has slightly distinct (low-keeled) dorsal ridges.
- Poorly developed distal part.

Malabaila

- Mixed hypendorcarp composition (horizontal fibres and vertical fibres).
- Well developed proximal part and swollen distal inflated part.

Tordylium (*Tordylium aegyptiacum*)

- The swollen wing margin and vesicular hairs on the epidermis.

Tordyliopsis brunonis

- Dorsal ribs (obsolete, widely distributed)
- Mixed hypendorcarp composition (horizontal fibres and vertical fibres).
- Proximal part features (thin, long). This is rather similar to *Tetrataenium* group.

Zosima

- The vittae almost occupy the entire areas of the mesocarp between each of the ribs. This is a very distinctive.
- Hypendocarp: not uniform, poorly lignified, various numbers of horizontal and vertical fibres

Heracleum millefolium

- Unique large rib secretory ducts,

Semenovia

- Dorsal ribs low-keeled, narrowly distributed
- Hypendocarp (mainly composed of horizontal fibres)

Fruit surface architecture (Chapter 7)

In order to identify further potentially useful taxonomic characters, the external architecture of the fruits was also investigated. Although this study is not complete, it is clearly shows that an examination of the micro-morphological characters of mericarp using SEM, particularly trichome type and surface pattern of trichomes, is very useful in delimiting generic groups. It can also give some potentially useful characters to indicate relationships within the component genera.

The diversity of mericarp surface structure found here is surprisingly varied and further work will have to be undertaken to consider variability and adaptive significances of the features of surface ornamentation.

Molecular (DNA) phylogeny

In order to evaluate the monophyly of Sino-Himalayan *Heracleum*, and to clarify the species relationships within this and related genera, the nuclear ITS and chloroplast rps16 intron and psbA-trnH spacer regions were sequenced for *Heracleum* and related genera (Chapter 8). Both ITS and rps16 intron regions show a good resolution of phylogenetic relationships down to species level, as well as good agreement between phylogenetic signal from the two regions. However, although psbA-trnH region has been successively tested in other plant families, it failed to resolve relationships among the major *Heracleum* clade. Only higher level resolution was successful. The comparative analysis confirmed the monophyly and refined the circumscription of the '*Heracleum* clade' *sensu* Downie. Within this clade several major lineages are defined (Fig. 8.1-13):

- *Heracleum*
- *Malabaila* + *Pastinaca*
- *Zosima*
- *Semenovia*
- *Tordylium*
- *Tordyliopsis*

The Sino-Himalayan *Heracleum* lineage is seen to comprise three distinct sub-lineages (*Heracleum* Groups A1, A2, and A3). These include the monotypic genus *Tordyliopsis* and the Central Asiatic genus *Semenovia*, which means that *Heracleum sensu lato* is not monophyletic. *Tetrataenium,* a generic segregate of *Heracleum* proposed in past classifications, can be equated with *Heracleum* Group A2 and A3.

These phylogenies also helped resolve the taxonomic placement of some enigmatic taxa such as *Heracleum millefolium* and *Tordylium.*

The previous sectional classification (Table 2.1) was compared with present study. Section *Heracleum* is well supported, but Sections *Villosa* and *Pubescentia* do not correlate well with the present study.

9.2 Evolutionary patterns of morphological characters

Among the morphological characters traditionally used in *Heracleum* and allies there are no perfect correspondences with the phylogeny presented in this thesis (Chapter 5, 6, 7). The same is also true of the morphological characters newly investigated in this study (Fig. 9.1 - 9.2).

Therefore there are few known constant synapomorphic characters that are taxonomically diagnostic within *Heracleum*. Exceptions include in petals yellow, equal and simple pinnate leaves in *Pastinaca-Malabaila* group. Although this is hampered by lack of complete sampling, even the sampling presented in this study suggests that only combined suites of several field characters together are useful for classification. These combinations of characters are shown in Fig. 9.1.

The evolutionary pattern of most character states seen across *Heracleum* probably reflects functional groupings rather than any taxonomically useful groups. For example, several Sino-Himalayan species (*H. nepalense*, *H. woodii*, *H. kingdonii*) and East European and West Asian species of *Pastinaca-Malabaila* group share triangular trichome type with papillate surface patterns even they are geographically, phylogenetically very distant group.

This lack of ideal morphological, taxonomically important characters remains a stumbling block to a useful field classification of *Heracleum* that reflects the true underlying phylogeny.

9.3 Biogeographical patterns of Sino-Himalayan *Heracleum*

Two main lineages have been identified within Sino-Himalayan *Heracleum*. These lineages are divided into sub-groups which correlated to geographical distribution (Fig. 8.1-8.2). Within one of these sub-group one distinctive species (*Heracleum lanatum*) presents a disjunctive distribution between Asia and eastern North America. Further work on this species near the Bering area will be needed. According to our result (phylogeny; see chapter 8) Himalayan taxa (*Tetrataenium* group) could be hypothesised as an ancestral distributional position of Chinese *Heracleum* and *Pastinaca-Malabaila* from East Europe and West Asia. However, for the complete estimation of the biogeographical patterns of *Heracleum,* DNA data for the Sino-Himalayan *Heracleum* and allies should be pooled with that for other regions, including the Caucasus and other parts of western Eurasia.

9.4 Synthesis of phylogeny and morphology – present and future work

The correlation between taxonomically useful morphological characters and the inferred molecular phylogeny resulting from this study are presented in Figure 9.1-9.2.

Heracleum

Corresponding to the 'core Heracleum group'. The sub-division of *Heracleum* (group 1, 2 and 3) was suggested by molecular results (chapter 8) and this supported by fruit micro-morphology characters (chapter 7)

- Fruit morphology: obsolete, widely distributed dorsal and lateral ribs, wing's narrower than fruit body
- Fruit anatomy: thick & short proximal part features,with short seed cavity, mixed hypendorcarp composition (horizontal fibres and vertical fibres).
- Fruit micro-morphology: trichome of ribbon (smooth) and cylindrical (papillate) shape

Tetrataenium

Previous classification concept of genus *Tetrataenium* (Sensus Mandenova) corresponds to the taxa in clades 2 and 3 in the phylogeny. This classification is supported by present study of general fruit morphology (chapter 5) and fruit anatomy characters (chapter 6). However, detailed SEM fruit micro-morphological study shows that this grouping can be divided into 3 different clades based on trichome types and trichome surface types (Chapter 7). This group would certainly include the members of *Semenovia* sampled for this analysis, but further research into the remainder of *Semenovia* (18 species in total) is required to

determine if the entire genus should be subsumed within *Tetrataenium*. The exact relationship between *Tetrataenium* and *Tordyliopsis* is unclear, but the morphology of *Tordyliopsis* suggests that it is a very distinct group. *Tordyliopsis* has glandular trichomes on the leaves, as well as many vegetative characters (e.g. distinctive bracts and bracteoles) were unique to this taxon. Further molecular data may well provide more evidence as to whether it is best treated as a monotypic genus or an infrageneric taxon within a broader *Tetrataenium*. All of the Sino-Himalayan members of *Heracleum* should be included in this group. South-east Asian species not included in this study certainly need to be included in further molecular investigations to determine if they fall within this group, but as DNA extraction from existing herbarium specimens was unsuccessful, extensive field research will be needed to gather appropriate new samples.

Tetrataenium: corresponds to the taxa in clades 2 and 3 in the phylogeny.

- Fruit morphology: dorsal ribs prominent, closely distributed dorsal and lateral ribs, wings equal or wider than fruit body
- Fruit anatomy: Proximal part features (thin, long) with relatively long seed cavity, vertical fibres dominant hypendorcarp composition.
- Fruit micro-morphology: trichome of ribbon shape (smooth), triangular (papillate), or trichome absent.

Heracleum millefolium

Heracleum millefolium is an enigmatic species which has yet to find a good generic placement. In recent treatments it has been variously included in *Heracleum* or *Semenovia* and there is no current consensus as to its placement. This high altitude Asiatic species has a very distinct morphology, with 3 to 4-pinnate basal leaves greatly reduced size and densely hairy fruits. All the

phylogenies inferred from the molecular studes are very interesting as they indicate that *Heracleum millefolium* is distinct from all other the *Heracleum* or *Semenovia* species so far sampled. Ideally further resolution of the polytomic 'backbone' of the phylogeny (Fig. 9.1) is required, but there is already a strong case for separating *H. millefolium* as a new genus.

- Fruit micro-morphology: ribbon shape trichome with papillate surface
- Fruit anatomy: Unique large rib secretory ducts,

Pastinaca + Malabaila

Malabaila is evidently close to *Pastinaca*, and the phylogenetic analysis of the current study did not provide a conclusive result regarding their monophyly relative to each other. Species from these genera were included in the present study were included as exemplars for these genera as focus of was primarily on Sino-Himalayan representatives. However, the results are intriguing and indicate that this pair of genera would form a very interesting study in themselves, For this a complete sampling from both genera (*Pastinaca* 4 species and Malabaila 15 species) and the examination of further gene regions would be needed to enable more definite taxonomic decisions to be made. As *Pastinaca* includes an economically important crop plant (Parsnip, *Pastinaca sativa*) such a study would be important in revealing close crop relatives for future crop development.

Pastinaca

- Fruit micro-morphology: triangular trichome with papillate surface
- Fruit anatomy: horizontal fibres and vertical fibres mixed hypendorcarp composition, slightly distinct (low-keeled) dorsal ridges, poorly developed distal part.

Malabaila

- Fruit micro-morphology: mixed hypendorcarp composition (horizontal fibres and vertical fibres), well developed proximal part and swollen distal inflated part.
- Fruit anatomy: well developed proximal part, distal inflated part (swollen).

Zosima

Zosima is distinct in morphology (Characters 6, 7), and also forms a well-supported clade in the phylogeny.

- Fruit micro-morphology: ribbon shape trichome with smooth surface, depressed stylopodium shape.
- Fruit anatomy: the vittae almost occupy the entire areas between each of the ribs, not uniform, poorly lignified, various numbers of horizontal and verticlal fibres

Tordylium

The taxonomic position of *Tordylium* has been contentious because previous classifications were wholly based on suites of morphological characters with no single character being diagnostic. The 18 species have been grouped together based on general morphology and fruit micro-morphological characteristics. The present phylogenetic and morphological analyses were limited to two and one exemplar species respectively. As in the case of *Pastinaca* and *Malabaila* discussed above, this is insufficient to make even a tentative taxonomic decision on the status of the genus, so further data from other taxa in *Tordylium* are required.

- Fruit morphology: smooth or corrugate fruit wing margins (Table 2.2)
- Fruit anatomy: the swollen wing margin and vesicular hairs on the epidermis.

Tentative classification

Basing generic delimitations using monophyly as a fundamental criterion, we can begin to construct the following tentative classification for Sino-Himalayan *Heracleum* and allied taxa.

Genera:

- *Heracleum*
- *Pastinaca* (may include *Malabaila*)
- *Zosima*
- *Tordyliopsis*
- *Tetrataenium* (to include all Himalayan species, and possibly *Semenovia*)
- Gen. nov. (*Heracleum millefolium* as a new genus)

Status uncertain:

- *Tordylium*

Future works

1. Phylogeny and DNA data thus far gathered for the Sino-Himalayan *Heracleum* / *Tetrataenium* and allies should be pooled with that for other regions, including the Caucasus and other parts of western Eurasia to produce a complete phylogeny and biogeographical distribution patterns for the group.

   Further field work throughout the range of the genus and its allies to ensure complete sampling would be necessary. This should focus on further work in China and other areas of Southern Asia, as these areas remain the least well sampled for *Heracleum* / *Tetrataenium*. These are often remote and difficult habitats to access, but it is essential to collect these from natural populations in the wild to fully understand the diversity of the genus and its chorological patterns.

2. Further research should also aim to increase the number of phylogenetically informative characters for each taxon sampled. Further gene regions should include those such as the chloroplast regions *trnD-T* and *trnS-G* that are known to be have relatively high rates of substitution.

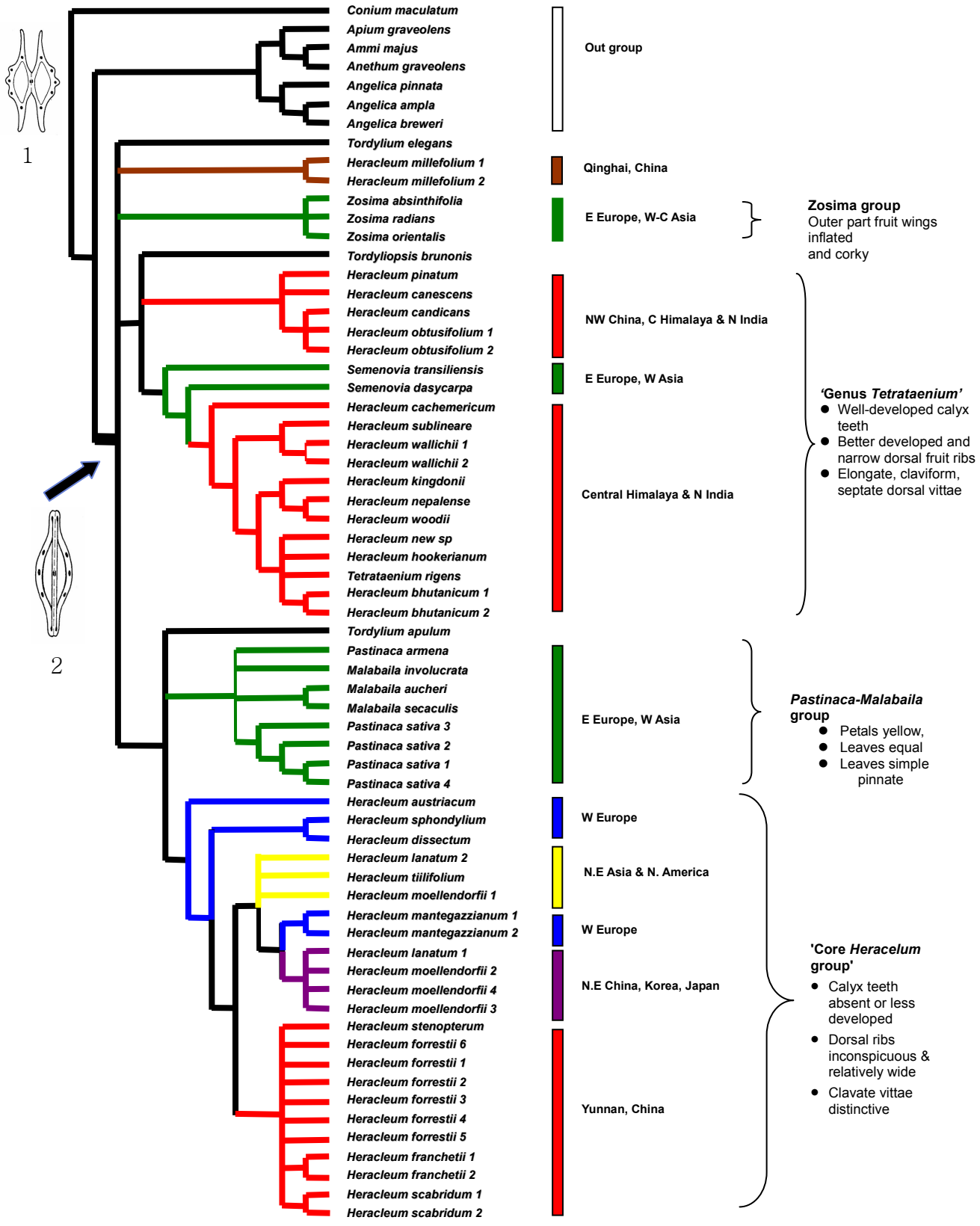


Figure 9.1 ITS strict consensus tree with general morphological characters.
Colours (bars & trees) represent biogeographical distribution patterns. Brackets indicate generic clades.
1: general fruit features of outgroup (two mericarps partly union); 2: general fruit features of ingroup (two mericarps completely union; Tribe Tordylieae including *Pastinaca*)

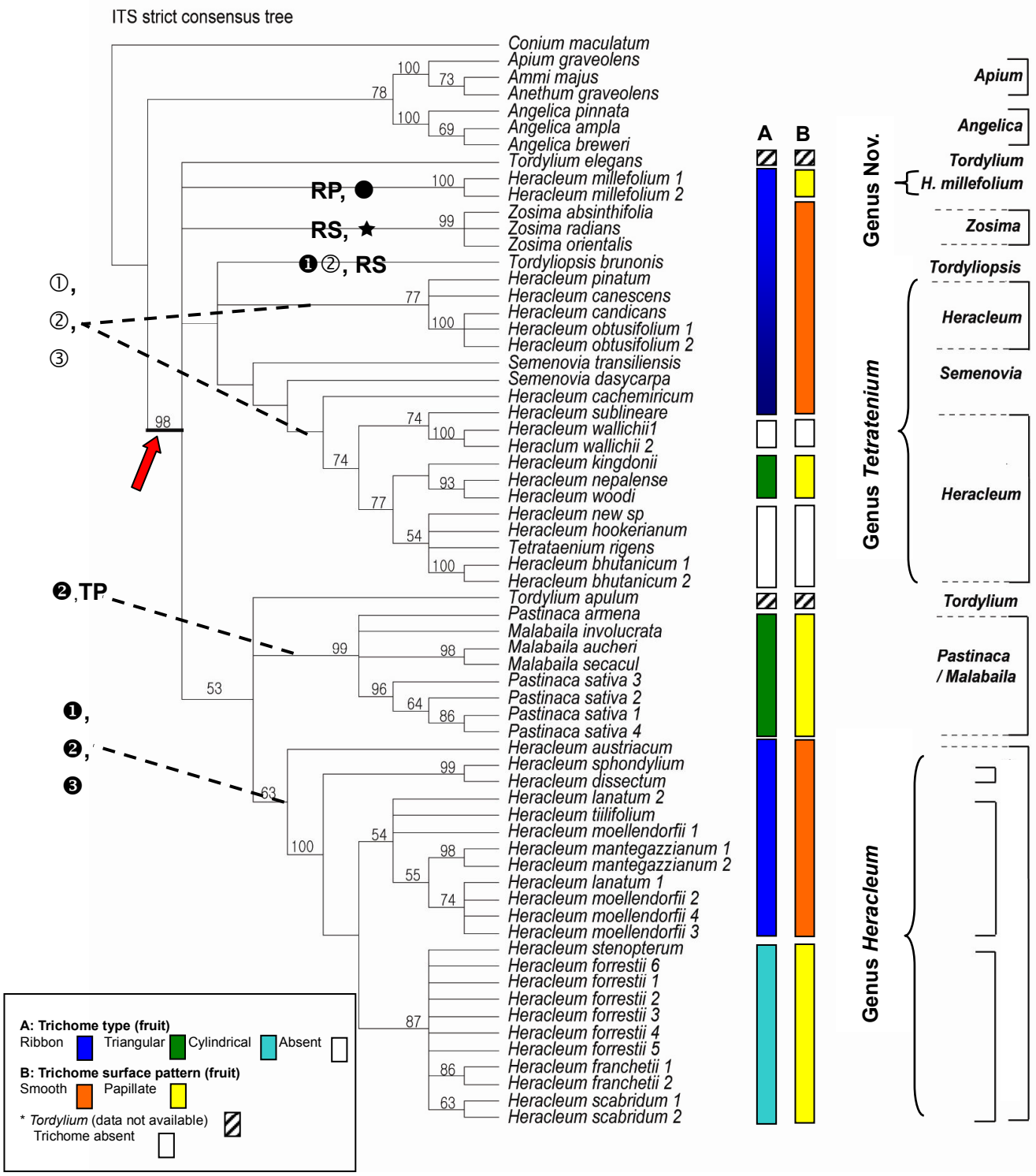


Figure 9.2 ITS strict consensus tree with micro- morphological characters. Brackets indicate generic or sectional separated clades.

❶: obsolete, widely distributed dorsal and lateral ribs, wing's narrower than fruit body

❷: thick & short proximal part features, with short seed cavity, mixed hypendorcarp composition (horizontal fibres and vertical fibres).

❸: trichome of ribbon (smooth) and cylindrical (papillate) shape.

①: dorsal ribs prominent, closely distributed dorsal and lateral ribs, wings equal or wider than fruit body

②: Proximal part features (thin, long) with relatively long seed cavity, vertical fibres dominant hypendorcarp composition.

③: trichome of ribbon shape (smooth), triangular (papillate), or trichome absent.

RP: ribbon shape trichome with papillate surface.

●: Unique large rib secretory ducts.

TP: triangular trichome with papillate surface.

❷**V**: horizontal fibres and vertical fibres mixed hypendorcarp composition, slightly distinct (low-keeled) dorsal ridges, poorly developed distal part.

▲: well developed proximal part, distal inflated part (swollen).

RS: ribbon shape trichome with smooth surface, depressed stylopodium shape.

★: the vittae almost occupy the entire areas between each of the ribs, not uniform, poorly lignified, various numbers of horizontal and verticlal fibres.

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http://www.floraofnepal.org

Appendix 1

Material used for DNA analyses with GeneBank accession numbers. V: GeneBank accession numbers not given.

Species	Source and Voucher	GeneBank accession No.		
		ITS	rps16 intron	psbA-trnH
Ammi majus L.	Downie *et al.* 1998	U78386+U78446		
Ammi majus L.	Downie *et al.* 2000		AF164814	
Anethum graveolens L.	Downie and Katz-Downie 1996	AH003470		
Anethum graveolens L.	Downie and Katz-Downie 1999		AF110542	
Angelica ampla A. Nelson	Downie *et al.* 1998	AH006065		
Angelica ampla A. Nelson	Downie *et al.* 2002		AF358598	
Angelica breweri A. Gray	Downie *et al.* 1998	U78396+U78456		
Angelica breweri A. Gray	Downie *et al.* 2002		AF358599	
Angelica pinnata S. Watson	Downie *et al.* 2002	AF358465	AF358600	
Apium graveolens L.	Downie and Katz-Downie 1996	AH003471		
Apium graveolens L.	Downie and Katz-Downie 1999		AF110545	
Conium maculatum L.	Downie *et al.* 1998	AH006070		
Conium maculatum L.	Downie and Katz-Downie 1999		AF110546	
Conium maculatum L.	Kress *et al.* 2005			DQ006135
Foeniculum vulgare Miller				AY581806
Heracleum austriacum L.	Austria, Salzburg 1400-1500m, M. Eysn *s.n.* (E)	EU185657	EU185707	V

Heracleum bhutanicum M.F.Watson	Bhutan, Chukka District, Chuka, 1400m, Watson 6811 (E)	EU185652	EU185702	V
Heracleum bhutanicum M.F.Watson	Bhutan, Chukka District, Chuka, 1500m, Wood 7158 (E)	EU185676		
Heracleum brunonis Benth. ex C.B.Clarke	Bhutan, Thimphu District, Talukah Gompa, 4200m, Wood 6616 (E)	EU185642	EU185692	V
Heracleum cachemericum Clarke	Pakistan, Punjab, Charihan Murree Hill 6000fit, Coventry *s.n.* (K)	EU185649	EU185699	V
Heracleum candicans Wall. ex DC.	China, Yunnan, Ace 1576 (E)	EU185643	EU185693	V
Heracleum canescens Lindl.	Nepal, Jumla. 7,600ft., Polunin et al 4461 (E)	EU185673		
Heracleum dissectum Ledeb.	Central Siberia, ca. 122km ESE of Novosibirsk, H.H. Iltis et al 1224 (NY)	EU185691		
Heracleum forrestii H. Wolff 1	China, Yunnan, Zhongdian County, Wu Feng Shan, Fed 34 (E)	EU185684		
Heracleum forrestii H. Wolff 2	China, Yunnan, Gongshan county, Gongshan to Kongdan, GSBS 16833 (E)	EU185685		
Heracleum forrestii H. Wolff 4	China, Yunnan, Zhongdian county, DaxueShan, 4100m, Fed 199 (E)	EU185688		
Heracleum forrestii H. Wolff 5	China, Yunnan, Gaoligongshan, GSBS 26517 (E)	EU185689		
Heracleum forrestii H.Wolff 3	China, Yunnan, Chungtien-plateau, 9000 fit, Forrest 13115 (E)	EU185687		
Heracleum forrestii H.Wolff 6	China, Yunnan, Gaoligongshan, Chukai, GSBS 31701 (E)	EU185660	EU185710	V
Heracleum franchetii Hiroe	China, Qinghai, Nangqên Xian, Ho et al 1752 (BM)	EU185661	EU185711	V
Heracleum franchetti Hiroe	China, Qinghai, Chindu Xian, 3600 m, Boufford *et al.* 26967 (E)	EU185690		
Heracleum hookerianum Wight & Arn.	India, Madras, 7000 fit, Gamble 16960 (K)	EU185653	EU185703	V
Heracleum kingonii H.Wolff	North Burma, Kachin state, The triangle, 4000 fit, Kingdon-ward 21305 (BM)	EU185675		
Heracleum lanatum Michx.	Japan, kyoto, Murata & Koyama 186 (E)	EU185656	EU185706	V
Heracleum lanatum Michx.	Downie *et al.* 1996	AH003476		
Heracleum lanatum Michx.	Downie and Katz-Downie 1999		AF110537	

Heracleum mantegazzianum Sommier & Levier 1	United Kingdom, Middle lothian, Drever 2 (E)	EU185658	EU185708	V
Heracleum mantegazzianum Sommier & Levier 2	United Kingdom, Middle lothian, Edinburgh, Mandenova *s.n.* (E)	EU185662	EU185712	V
Heracleum millefolium Diels	China, Qinghai, Yushu Xian, 3850-3950 m, Ho et al 2087 (E)	EU185655	EU185705	V
Heracleum millefolium Diels	China, Qinghai, Madoi Xian, 4070m, Ho et al 1565 (BM)	EU185670		
Heracleum moellendorffii Hance 1	China, Anhoui? . Alt. 1200m, Zhongwen & Lin 97097 (MO)	EU185681		
Heracleum moellendorfii Hance 2	Choi, *et al.* 2004	AY548226		
Heracleum moellendorfii Hance 3	Korea, Kangwon province, Lee 0013861 (KWNU)	EU185682		
Heracleum moellendorfii Hance 4	Korea, Kangwon-do, Lee 0053777 (KWNU)	EU185683		
Heracleum nepalense D. Don	Bhutan, Upper Mochu district, 3850 m, Sinclair et al 5342 (E)	EU185651	EU185701	V
Heracleum obtusifolium Wall. ex DC.	Bhutan, Thimphu, Wood 7034 (E)	EU185644	EU185694	V
Heracleum obtusifolium Wall. ex DC.	China, Qinghai, yushu Xian, 3600 m, Ho et al 2339 (BM)	EU185671		
Heracleum pinnatum C.B.Clarke	India, Padum, Zanskar district, Jammu and Kashmir State, Grace *et al.* 3 (K)	EU185672		
Heracleum scabridum Franch.	China, Yunnan, Forrest 2260 (E)	EU185667	EU185721	
Heracleum scabridum Franch.	China, Yunnan, Yangtze watershed, Likiang, Rock 5631 (E)	EU185686		
Heracleum sphondylium L.	United Kingdom, Whalsay, Beanell 13/7 (E)	EU185663	EU185713	V
Heracleum spp. (new)	China, Yunnan, Gaoligongshan, Chukai, GSBS 31616 (E)	EU185650	EU185700	V
Heracleum stenopterum Diels	China, Yunnan, 10-11000ft, Forrest 2847(E)	EU185659	EU185709	V
Heracleum sublineare C.B.Clarke	Bhutan, Sankosh District, 3600m, Wood 7058 (E)	EU185654	EU185704	V
Heracleum sublineare C.B.Clarke	Nepal, Bheding, 12000-13000ft, Dhwoj 272 (E)	EU185677		
Heracleum tillifolium H.Wolff	China, Hupei, Yang Long co., Long Kan village, Ye Cunsu 9603 (MO)	EU185680		

Heracleum wallichii DC.	Nepal, Malemchi, 7500ft, Stainton 6666 (BM)	EU185678		
Heracleum woodii M.F. Watson	Bhutan, Thimphu District: Phajoding, Wood 7138 (E, sheet 3)	EU185674		
Malabaila aucheri Boiss.	Iran,D5 (Times Atlas): 10km. E. of Bebehan, Davis & Bokhari 55816 (E)	EU185665	EU185716	v
Malabaila involucrata Boiss. & Spruner	Greece, Roadside at 600m, Cghanson *s.n.* (E)	EU185669		
Malabaila secacul (Banks & Sol.) Boiss.	Downie *et al.* 2000		AF164802	
Malabaila secacul (Banks & Sol.) Boiss.	Katz-Downie *et al.* 1999	AF008627		
Pastinaca armena Fisch. & Mey.	Katz-Downie *et al.* 1999	AF008626		
Pastinaca armena Fisch. & Mey.	Downie *et al.* 2000		AF164803	
Pastinaca sativa L.	Downie *et al.* 1996	AH003484		
Pastinaca sativa L.	Bulgaria, Sofia, Rila Planina, Musala, 1250m, Gardner & Gardner 3202 (E)	EU185666	EU185717	V
Pastinaca sativa L.	USA, North Carolina, Ashe County, Pence 44875 (E)	EU185668		
Pastinaca sativa L.	Downie and Katz-Downie 1999		AF110538	
Semenovia dasycarpa Korov. ex Pimenov & V.N.Tikhom.	SE Afghanistan, Orozgan: Inter Tirin, 2000m, K. H. Rechinger 35159 (G)	EU185645	EU185695	V
Semenovia transiliensis Regel & Herd.	Kazachstania, declivitas borealis jugi Kungei-Alatau, I. Roldugin *s.n.* (NY)	EU185646	EU185696	V
Tetrataenium rigens (Wall. ex DC.) I.P.Mandenova	Downie *et al.* 1996	AH003477		
Tetrataenium rigens (Wall. ex DC.) I.P.Mandenova	Downie and Katz-Downie 2000		AF164804	
Tordylium Apulum L.	Turkey, B1 Izmri, cesme 10-50m, Davis 41835 (E)	EU185679		
Tordylium Elegans (Boiss. & Balansa) Alava & Hub.-Mor.	Turkey, Adana, Alava 6694 (E)	EU185664	EU185714	

Zosima absinthifolia DC.	Azerbaijan,between khiyav and Ahar, 1100m, Lamond 3327 (E)	EU185647	EU185697	V
Zosima orientalis Hoffm.	Katz-Downie *et al.* 1999	AF008628		
Zosima orientalis Hoffm.	Downie *et al.* 2000		AF164806	
Zosima radians Boiss. & Hohen.	Iran, Tehran, Between Tehran and Karadj, Assadi & Mozaffarian 27560 (E)	EU185648	EU185698	V

Appendix 2

Running head: Phylogeny of Sino-Himalayan *Heracleum* and related genera

Phylogenetic relationships of Sino-Himalayan *Heracleum* L. (Umbelliferae/Apiaceae subfamily Apioideae) and related genera.

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Abstract

The taxonomic delimitation of and within the genus *Heracleum* L. (Apiaceae/Umbelliferae) has long been problematic. Recent molecular phylogenies have provisionally identified a well supported " *Heracleum* clade" *sensu* Downie within the Apioid superclade which includes *Heracleum* and related genera. *Heracleum* has been revised in the past giving rise to several controversial classifications based primarily on morphology, but as yet the Sino-Himalayan species of *Heracleum* have not received detailed study from modern molecular methods. DNA sequence data of ITS and rps16 intron of Sino-Himalayan *Heracleum* and related genera were studied using Maximum Parsimony and Bayesian Analysis to determine the monophyly of *Heracleum* and clarify species relationships within this group. Both ITS and rps16 intron regions show good resolution of phylogenetic relationships down to species level. The results of the comparative phylogenetic study confirmed that the "*Heracleum* clade" sensu Downie remains monophyletic. Within this clade at least six major lineages are defined: *Heracleum*, *Malabaila-Pastinaca*, *Zosima*, *Semenovia*, *Tordylium* and *Tordyliopsis*. The Sino-Himalayan *Heracleum* lineage is seen to comprise two distinct sub-lineages (*Heracleum* Groups I & II) including several European *Heracleum* species and with the monotypic genus *Tordyliopsis* and Central Asiatic genus *Semenovia*. *Heracleum* itself is thus not monophyletic. Generic segregates of *Heracleum* proposed in past classifications, such as *Tetrataenium,* can be equated with these subclades and are thus supported. The taxonomic placement of some enigmatic taxa such as *Heracleum millefolium* is partially resolved and further comments on the relationships of *Tordylium* can be inferred.

KEY WORDS: Phylogeny, ITS, rps16 intron, Maximum Parsimony, Bayesian Analysis

Introduction

In northern temperate regions the genus *Heracleum* L. is a familiar representative of the Umbellifer family (Apiaceae/Umbelliferae). These often large, robust plants with dorsally compressed flat fruit and distinctive short clavate vittae are easy to spot and some, such as the massive Giant Hogweed, *Heracleum mantegazzianum* (Brummitt, 1968; Jahodová & al., 2007), are notorious both as invaders and for the sap which can photosensitise the skin causing blistering in bright sunlight. Worldwide the genus *Heracleum* includes 65-70 species, and is distributed right across the northern hemisphere from North America to East Asia (Pimenov & Leonov, 1993). They are usually perennial or rarely biennial herbs and can be adapted to wet or dry habitats, but usually occur in regions of temperate forests, particularly in upland areas. The taxonomy of *Heracleum* presents some complex and interesting problems because of the variety of morphological types and the lack of clear boundaries between many of the species. Unlike the majority of Umbellifers, hybridisation also plays a part in confusing taxon delimiation (Stewart & Grace, 1984) but this appears to be confined to *H. sphondylium* L. and it close relatives. Even the delimitation of the genus is not straightforward as deviations from the classic European forms occur in Asia. The distinctive clavate vittae, shorter than the length of the mericarp, so characteristic of many *Heracleum* species in the western parts of Eurasia (Brummitt, 1968), breaks down in SE Asia and generic limits both within and with several other genera (*Angelica*, *Peucedanum* and *Semenovia*) are blurred (Hedge & Lamond, 1992). Past authors have attempted to revise *Heracleum* using morphological and anatomical data and several controversial classifications have been proposed adding to the nomenclatural tangle (Briquet, 1924; Kowal, 1975; Mandenova, 1950) and giving rise to segregate genera and many infrageneric and infraspecific taxa. *Semenovia* is

a Central Asiatic genus of 18 species, sometimes included within and sometimes separated from *Heracleum* (Pu & Watson, 2005; Watson, 1999). The monotypic Himalayan genus *Tordyliopsis* is also sometimes included within *Heracleum* (Pimenov & al., 2000). However, the separation or inclusion of these segregate genera is problematic in many cases because these treatments did not take account of the high variation in characters used. Additionally, good quality herbarium specimens and full documentation are lacking in many instances.

High level molecular phylogenies have provisionally identified a '*Heracleum* clade' within the Apioid superclade comprising: *Tetrataenium*, *Tordylium*, *Heracleum* and *Pastinaca*, and possibly *Conium*, *Malabaila* and *Zosima* (Katz-Downie & al., 1999; Downie & al., 2000a & b; Downie & al. 2001). Among their '*Heracleum* clade' only *Heracleum* has representatives in the Sino-Himalayan region (Nepal eastwards to SW China), and the sampling within *Heracleum* has so far been rather limited. However, the Sino-Himalayan region is very important to the understanding of *Heracleum* and its related genera as there are ten *Heracleum* species in the Himalaya, and about 30 species in China The East Himalaya/SW China region is one of two centres of diversity of this widespread genus, the Caucasus region being the other. Also, problems of morphological complexity and blurred generic boundaries in this region highlight its importance. Very recently, Logacheva & al. (In press) sampled the Western Asiatic taxa and suggested that *Heracleum* forms a polyphyletic clade which has poor concordance with previous classifications.

De Candolle (1829) was the first to include Himalayan species in his taxonomic revision of *Heracleum* where he divided the 26 species known at that time into six sections based on the number of commissural vittae of the fruit. He included four Himalayan *Heracleum* species, placing them in two new sections: Tetrataenium: *H. nepalensis*, *H. obtusifolium*, *H. regense* and Trichogonium: *H. wallichii*. Section Trichogonium was not used in later revisions, but Section Tetrataenium was elevated to generic rank by Mandenova (1959,1978, 1982) include these and other species mainly from Central Himalayan and South India. The first complete treatment of *Heracleum* in the Central Himalaya and India was the detailed account by Charles Baron Clarke (1879) in J. D. Hooker's 'Flora of British India'. His account of *Heracleum* recognized 21 species distinguished primarily using characters of gross morphology and fruit. Since this classic work, members of the genus have been treated in local Floras and a number of new taxa have been reported. Important contributors for the Himalaya region include Gamble (1919), H. Wolff (1910-1930), Nasir (1972), Cannon (1978) and Watson (1999). The most recent assessment of *Heracleum* covering the whole of India and Central Himalaya region is 'Umbelliferae of India' by Mukherjee & Constance (1993). In this account 20 *Heracleum* species were recognized and *Tetrataenium* was included within *Heracleum*. The authors decided that the characteristics of *Tetrataenium* (Mandenova, 1987) are not clearly defined and much more population level investigations were needed.
The classification of Chinese *Heracleum* has been attempted several times, initially by European botanists using rather limited herbarium materials (e.g. Franchet 1894, de Boissieu 1903, and Wolff 1933-34). Chinese regional Floras did not make significant contributions to the taxonomy of *Heracleum* in China until the publication of 'Flora Reipublicae Popularis Sinicae' (Shan & Sheh, 1992) and the slightly modified and revised treatment for 'Flora of China' (Pu & Watson, 2005). In these works *Heracleum* was revised for parts of the range of the genus and almost all the species were well illustrated, however, they mostly followed a broad generic circumscription, not recognising the segregate genera. He *& al*. (1998) published a revision of Chinese *Heracleum* and for the first time used fruit morphology and anatomy combined with pollen morphology, cytology and anatomical characters of petiole.

They divided the 29 Chinese *Heracleum* into five sections (Sect. Wendia, Sect. Heracleum, Sect. Villosa, Sect. Millefolia and the new Sect. Plurivittata).

The current study is aimed at extending previous molecular studies to include representatives of *Heracleum* and related genera from the Sino-Himalayan region to assess these past taxonomic treatments and establish a robust phylogeny. Our first objective is to evaluate the monophyly of *Heracleum*, determine the validity and monophyly of segregate genera, and determine species relationships. The second objective is to evaluate relationships between related allies of *Heracleum* included in the 'Heracleum clade' as a basis for future research.

Materials and Methods

Molecular tools

The suitability of the nuclear ITS region and the rps16 chloroplast region to resolve inter- and intra- generic phylogenies has already been successfully shown in a range of studies on Apiaceae (Downie & Katz-Downie 1996, 1999; Downie & al. 1998, 2000a, 2000c; Lee and Downie 1999, 2000) as well as in angiosperms in general (Baldwin & al. 1995; Oxelman & al., 1997). In these studies both regions have resolved phylogenetic relationships down to species level.

Taxon sampling

On the basis of previous studies (Katz-Downie & al., 1999; Downie & al., 2000a & b; Downie & al. 2001) *Conium maculatum*, *Apium graveolens*, *Ammi majus*, *Anethum graveolens*, and three *Angelica* species were used as outgroups. Complete ITS1 and ITS2 sequences for 50 taxa are reported here for the first time; combining with 14 previously published ITS sequences (Downie and Katz-Downie, 1996) in a matrix of 64 taxa. For the rps16 intron comparative analysis, 27 sequences produced as part of this investigation were combined with 12 already published (Downie and Katz-Downie, 1996) resulting in a data matrix of 39 taxa. In addition, sequenced data for the psbA-trnH cpDNA intergenic spacer were obtained for 24 taxa. The 24 new complete psbA-trnH spacer sequences were combined with 2 previously published sequence obtained from GeneBank. Voucher specimen information and accession numbers are listed in the Appendix. The plants were identified based on published Floristic accounts and comparison with verified herbarium specimens.

DNA extraction, Amplication, and Sequencing

Leaf material for DNA extraction was obtained either directly from the field (dried using silica gel) or from herbarium specimens. Total DNA was isolated from leaf tissue using the DNeasy Plant Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacture's protocol. Extracted DNA was used as a template for polymerase chain reaction (PCR) for ITS, rps16 and psbA-trnH sequencing.

ITS region

PCR amplification of the ITS region, excluding the 5.8S rDNA region, was performed using primers ITS-1 and ITS-4 (White & al., 1990). Additional primers ITS-5 (5' GGAAGGAGAAGTCGTAACAAG 3': manufactured by SIGMA), ITS-8 (5' TGTAGTCTGGAGAAGCGTG 3': manufactured by TAGN) were used for this study, as primers ITS-1 and ITS-4 failed to generate PCR products for some taxa.

PCR was performed on a MJ Research PTC-200 Thermal Cycler in 50 $\mu\ell$ reaction; using reagents from Bioline (U.K.). Final reaction volume of 50 $\mu\ell$ reaction using reagents from

Bioline (U.K.). Reactions contained: 5.0 µl of 10x NH_4 buffer, 5.0 µl of 0.2 mM dNTPs, 2.5 µl of 50mM $MgCl_2$, 2 µl of each 10 µm primer, 0.125 units of Biotaq polymerase and 1 or 2 µl of DNA. Amplification for ITS regions 1 and 2 were carried out using 1 cycle at 94℃ for 3 min, 40 cycles of 1 min at 94℃, 1 min at 52℃, 2 min at 72℃ and then a final cycle at 72℃ for 2 min. prior to sequencing, the 50 µl reaction were pooled together and purified using either Qiaquick MinElute PCR purification kit (Qiagen Ltd., Crawley, West Sussex, UK) or GFX PCR DNA Purification Kit (Amersham Biosciences, UK).
The sequencing reactions were performed using the purified PCR products and were carried out in 20 µl reactions containing 1 µl primer (10mM), 1-2 µl purified PCR products, 13-14 µl dH_2O and 4 µl DTCS Quickstart mix (Beckman Coulter).
Sequencing reaction conditions were: 1 cycle of 96℃ for 2 min, 50℃ for 20 sec of 35 cycles and 60℃ for 4 min.

All sequenced products were purified by ethanol precipitation to remove unincorporated labelled ddNTPs and excess salts following the protocol of the manufacturer. All sequences were read on a Beckman Coulter CEQ™ 8000 Sequencer, then edited manually using SEQUENCHER V.4.1.4 (Gene Codes Corporation 2002).

Rps16 intron
The rps16 intron gene was performed using PCR primers *rps16_F* (5' AAACGATGTGGTARAAAGCAAC 3': Shaw & al., 2005) and *rps16_R* (5' AACATCWATTGCAASGATTCGATA 3': Shaw & al., 2005). PCR reaction conditions were identical as described for ITS region.

Sequence analysis
All sequences were aligned manually in the data editor of PAUP* version 4.0β (Swofford 2001), with gaps positioned to minimize nucleotide mismatches. Indels of more than one base were coded as the same presence/absence character state if they could not be interpreted as separate duplication or insertion events. In several regions, gap coding was problematic because of homopolymers or indirect duplications of adjacent elements in two or more taxa. These gaps were not scored and these ambiguous regions were excluded from subsequent analyses. Boundaries of the genes ITS and rps16 were determined by comparison of the DNA sequences to corresponding boundaries in other Apiaceae (Downie & al. 2000a, b). The nucleotide sequence data reported in this study have been deposited in the GenBank Library; accession numbers are provided the Appendix. The alignments of these sequences can be obtained directly from the authors.

Phylogenetic analysis of molecular data
Three sets of phylogenetic analyses were undertaken. Initially, a maximum parsimony analysis of ITS data for all 64 taxa was carried out to test the monophyly of the "*Heracleum* clade" and placement of the Sino-Himalayan *Heracleum* and related genera. The second analysis of a smaller matrix from the rps16 intron used maximum parsimony separately (39 taxa) and in combination with ITS data.

To examine the extent of conflict among separate data sets, the incongruence length difference test (Farris & al. 1994) was conducted using the partition homogeneity test within PAUP*. One hundred replicates were considered for each partition, using simple addition sequence of taxa and tree bisection reconnection (TBR) branch swapping. Incongruence

among data sets is identified if the additive tree lengths taken from resampled matrices are greater than the sum of the tree lengths from the original data.

Nucleotide sequences were analysed using parsimony. Analyses were performed using PAUP* 4.0β10 (Swofford 2001). Heuristic search options included an initial heuristic search using 10,000 random addition replicates, tree-bisection-reconnection (TBR) branch swapping with steepest descent off and Multrees activated. Trees produced from this analysis were used as the basis for a further heuristic search, activating steepest descent and Multrees and saving 10,000 trees which is considered to be sufficient to capture all topological variation. To limit the number of trees found, the "collapse branches if minimum length is 0" option was implemented. As an approximate guide to clade support, bootstrap values (Felsenstein 1985) were calculated for the three combinations of analyses using 10,000 bootstrap replicates each heuristic search on one random addition replicate, and TBR branch swapping. Steepest decent and Multrees were not activated.

Bayesian inference of the separate and combined ITS and rps16 intron (indel characters included) were conducted using MrBayes vers. 3.1.1 (Huelsenbeck and Ronquist, 2001). Prior to analysis, Modeltest 3.6 (Posada and Crandall 1998) was used to select evolutionary model of nucleotide substitution that best fits each partition in the data as selected by the Akaike Information Criterion estimator. The settings appropriate for the best-fit models were put into a MrBayes block in PAUP*. From different random starting trees, four independent Bayesian analysis (nruns=4) were run for 10 million generations and the trees saved to a file every 1000 generations. Four simultaneous Markov Chain Monte Carlo (MCMC) chains were used and branch lengths of the trees were saved. The states of the chain that were sampled before stationarity (i.e., the "burn in" of the chain) were discarded (25% proportion discarded after checking stationarity) and the posterior probability values for each bipartition of the phylogeny were determined from the remaining trees.

Results

Sequence characteristics

ITS sequence characteristics

Among the 64 species examined for ITS sequence variation, the length of the ITS 1 and ITS 2 regions varied from 420 to 443 base pairs. Data from the intervening 5.8S region were excluded because newly sequenced species were invariable in this region and several existing GenBank accessions were missing the 5.8S region. The alignment of complete ITS sequences resulted in a matrix of 616 positions. Of these, 197 were excluded from subsequent analyses because of alignment ambiguities, resulting in a matrix of 419 unambiguously aligned positions. The ambiguous positions represented tracts of poly-As, -Gs or –Ts, or indirect duplications of adjacent elements in two or more taxa. Characteristics of all unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 1. Unambiguous alignment gaps were potentially parsimony informative, ranging from 2 to 3 bp in size. Pairwise sequence divergence ranged from 0 (identical) to 18 % of nucleotides within the ingroup, and ranged from 11.3% to 24.3% between the outgroup and ingroup respectively.

Rps 16 intron sequence characteristics

The 39 species examined for rps16 intron sequences varied from 783 to 878 bp. The alignment of 39 complete rps16 intron sequences resulted in a matrix of 966 positions. Here, 93 positions were excluded from further consideration due to ambiguity of alignment.

Characteristics of all unambiguous positions, including the number of constant, autapomorphic, and parsimony informative sites, are presented in Table 1. Sixteen unambiguous alignment gaps were potentially parsimony informative, range from 5 to 22 bp in size. Pairwise sequence divergence ranged from 0 (identity) to 2.85 % of nucleotide within ingroup, and ranged from 1.03% to 4.49% of nucleotide between outgroup and ingroup respectively.

ITS and rps16 intron combined sequence data

A data set of 39 taxa for which both ITS and rps16 intron sequence data were available were included in a combined analysis. The partition homogeneity test between the ITS and rps16 data sets gave a value of P=0.129, reflecting no significant incongruence between data sets. Similarly, a review of bootstrap support values between the separate ITS and rps16 intro topologies showed no incompatible clades regardless of the bootstrap support and therefore, it was deemed acceptable to combine both these data sets.

Phylogenetic Analysis

1. ITS PHYLOGENETIC ANALYSIS AND RESULTS

Maximum parsimony analysis of all 419 unambiguously aligned positions resulted in 128 most parsimonious trees, each 584 steps long, with consistency indices of 0.611 (0.521 without uninformative characters), and a retention index of 0.793 (Table 2). The strict consensus of these trees is not fully resolved, but shows better resolution when gap characters were included in the analysis. Some branch support values were also increased as a result. The topology of the Bayesian AIC majority rule consensus tree, calculated using a GTR+G model, is almost identical to the MP strict consensus tree. The posterior probability values (pp) for the different clades were generally higher than the bootstrap supports.

Monophyly of the '*Heracleum* Clade' *sensu* Downie.

Trees inferred from maximum parsimony and Bayesian analyses are almost identical though the Bayesian trees have generally higher posterior probability values for the different clades than the bootstrap supports.

One main clade is recognized comprising of *Heracleum*, *Pastinaca*, *Malabaila*, *Zosima*, *Tordylium*, *Semenovia*, and *Tordyliopsis*. (Fig.1, 2) This clade forms strongly supported monophyletic group. This result corresponds to previous works (Katz-Downie & al., 1999; Downie & al., 2000a & b; Downie & al. 2001) where this group has been recognized as the '*Heracleum* Clade'.

Polyphyly of Sino-Himalayan *Heracleum*

Within the '*Heracleum* Clade' at least six distinct clades are recognized (Fig. 1.), that correspond to approximate generic groupings with several unresolved taxa (*Semenovia*, *Tordylium*, *Tordyliopsis*, and *Heracleum millefolium*). *Heracleum* is included in at least two distinct clades, so it does not appear to be monophyletic. *Heracleum* species are widely distributed throughout the main '*Heracleum* Clade' and separate lineages have been identified as *Heracleum* Group I and *Heracleum* Group II.

***Heracleum* Group I:** The clade with the largest number of taxa, is not strongly supported with *H. austriacum* is sister to all the other species. However, without *H. austriacum*, this group is more strongly supported (100% bootstrap). This clade can be divided into three sub-clades (A1, A2, and A3). Sub-clade A1 is well supported and consists of *Heracleum scabridum*, *H. franchetii*, *H. stenopterum* and *H. forrestii*. Accessions of *Heracleum forrestii*

and *H. stenopterum* are identical and only one base pair different to *H. franchetii* and *H. scabridum*. *Heracleum moellendorfii*, *H. lanatum*, *H. mantegazzianum*, and *H. tiliifolium* comprise a sub-clade A2. This clade is also not fully resolved and only weakly supported. However, *H. mantegazzianum* is strongly clustered as sister to the *H. moellendorfii* group. *Heracleum moellendorfii* and *H. lanatum* show paraphyletic and the relationships are unclear. Strongly supported sub-clade A3 consists of *H. sphondylium* and *H. dissectum* as sister to all other sub-clades.

***Heracleum* Group II:** The monotypic *Tordyliopsis*, *Semenovia* (2 species) and *Heracleum* (17 accessions) comprise a distinct but not well supported clade (bootstrap value less than 50%). The species of genus *Heracleum*, except *Semenovia* and *H. cachemericum,* is divided into two sub groups (sub clade B1, B2), and the monophyly of each is strongly supported. Sub clade B1 is mainly of central Himalayan and south Indian taxa. *Heracleum wallichii* and *H. sublineare* form a monophyletic group which is sister to other species. *Heracleum kingdonii*, *H. nepalense* and *H. woodii* comprise poorly supported group. The other species from south India (*Tetrataenium rigens (=H. rigens)*, *H. hookerianum*), Bhutan (*H. bhutanicum*) and a potentially new *Heracleum* species (from Chukai, Yunnan Province) comprise a recognisable group, but the relationships among these species are unclear owing to the unresolved areas in the strict consensus tree. *Heracleum pinnatum*, *H. canescens*, *H. candicans* and *H. obtusifolium* comprise moderately supported subclade B2. *Heracleum candicans* and *H. obtusifolium* forms a monophyletic (100% bootstrap) group sister to *H. pinnatum* and *H. canescens*.

It is interesting to note that genus *Semenovia* and *Tordyliopsis* arise within *Heracleum* Group II. The two accession of *Semenovia* (*S. transiliensis*: Kazachstania, *S. dasycarpa*: SE Afghanistan) are more closely related to sub clade B2 (Fig. 2). The placement of *Tordyliopsis* is not well resolved, however, separate and combined analyses of ITS and rps16 show that it is more likely to be sister to sub clade B1.

***Pastinaca-Malabaila* Group**
The genera *Pastinaca* and *Malabaila* comprise a strongly supported monophyletic group (99% bootstrap) sister to *Heracleum* Group I. In our analyses species of *Pastinaca* and *Malabaila* are generally separated, however, the position of *P. armena* and *M. involucrata* are not clear because these are not resolved.

***Zosima* Group**
All representative species of *Zosima* (*Z. absinthifolia*, *Z. radians*, and *Z. orientalis*) comprise a well supported monophyletic group (99% bootstrap) sister to all other members of the *Heracleum* group II. Unfortunately resolution within this clade is very poor.

Heracleum millefolium* and *Tordylium
These analyses go some way towards resolving the taxonomic placement of the morphologically distinct but taxonomically enigmatic *Heracleum millefolium* and *Tordylium* species. The two *Heracleum millefolium* accessions are fall outside any of the other *Heracleum* groups, but form an isolated well supported clade (100% bootstrap). Rather surprisingly the two species of *Tordylium* appear in different places within the main *Heracleum* clade although neither appears related to any of the included groups. The exclusion of these two taxa did not change of the overall topology of the tree.

2. RPS16 PHYLOGENETIC ANALYSIS AND RESULTS

Maximum parsimony analysis of 878 unambiguously aligned rps 16 intron nucleotides plus 9 binary-scored informative gaps, with gap states treated as missing data, resulted in 6 most parsimonious trees. Each trees was 151 steps long, with consistency indices of 0.781 and 0.467 (without uninformative characters), and a retention index of 0.851, of which their strict consensus (with accompanying bootstrap values). The Bayesian majority rule consensus tree (not presented), calculated using TVM+I+G model, is mostly congruent to the MP strict consensus tree. The posterior probability values (pp) for the different clades were generally higher than the bootstrap supports. This strict consensus tree (not presented) is generally congruent to that obtained in the analyses using a broader sampling of ITS nucleotides (Fig 1). In the strict consensus tree four accessions (*Conium*, *Apium*, *Ammi*, and *Anethum*) comprise outgroups to the other clades. However, it is interesting to note that the *Angelica* species group together (92% bootstrap) but are no longer outgroup to the main *Heracleum* clade but form a close sister group to other clades.

3. ITS AND RPS16 INTRON COMBINED DATA ANALYSIS AND RESULTS

The parsimony analysis of the 1310 parsimony informative characters from the combined ITS and rps16 intron sequences yielded 343 most parsimonious trees with a tree length of 667, a consistency index of 0.663 (0.484 excluding uninformative characters) and a retention index of 0.764. Their strict consensus is presented in Fig 2. The topology of the majority rule consensus tree of the Bayesian Analysis (calculated using SYM+G (ITS), TVM+I+G (rps16) model separately) was nearly identical. The posterior probability values (pp) for the different clades were generally higher than the bootstrap supports and ranged from 0.60-1.00. In the strict consensus tree the *Angelica* species form a well differentiated clade and again part of the outgroup, but close sister to the monophyletic main '*Heracleum* clade'.

DISCUSSION

Sampling

Central Himalayan species of *Heracleum* are well covered, but only 13 of a possible 27 species from SW Chinese (Pu & Watson, 2005) could be included. A major problem for molecular studies is that many *Heracleum* species recorded from SW China are only known from old historical collections which are in very poor condition and too old for molecular study: many specimens were sampled but the DNA was found to be too degraded. Another problem is that many of these older specimens are not representative of these large plants and so species identification is often ambiguous. Therefore, Chinese taxa had to be carefully selected to only include those with clear identification and material with DNA in good enough condition for sequencing. The *Heracleum* species from Southern Indian, SE Asia (Myanmar and Thailand) also not included in the molecular analysis are also only known from very old material and again DNA samples from these herbarium specimens was found to be too degraded. Although fieldwork was undertaken to parts of SW China to study population level variation and to collect fresh herbarium and DNA material, an extensive series of expeditions would be required to complete the sampling in SW China and the other areas in Asia.

Monophyly of the main *Heracleum* clade *sensu* Downie

The *Heracleum* Clade *sensu* Downie previously identified within the Apioid super clade comprising: *Heracleum*, *Pastinaca*, *Malabaila*, *Tetrataenium*, *Zosima*, *and Tordylium* (Katz-Downie & al., 1999; Downie & al., 2000a & b; Downie & al. 2001). These studies outlined the content of the *Heracleum* Clade, but were limited by sampling (10 species) and were provisional as the rps16 analysis results did not support monophyly of this clade with problems of equivocal position of *Tordylium* (Downie & al., 2000a). Our study with a much

enlarged taxon sampling confirms the previous circumscription of the '*Heracleum* Clade' with addition of *Semenovia* and *Tordyliopsis*. The result from the present study, the combined and separate analysis of ITS, rps16, and psbA-trnH (the result is not shown here), have confirmed that *Heracleum* clade is monophyletic (Fig. 1, 2). This clade is very strongly supported by MP (93 -100% bootstrap support) and BA analysis (100% posterior probability).

Our study have shown that '*Heracleum* Clade', with the addition of *Pastinaca*, *Semenovia* and *Tordyliopsis* correspond to Tribe Tordylieae W.D.J. Koch (=Drude's Peucedaneae subtribe Tordyliinae), including eight of the 23 genera recognized within Tordylieae (Pimenov and Leonov 1993). Monophyly of Tribe Tordylieae and its separation from other Peucedanoid taxa has been previously supported by Shneyer & al. (1995), using seed-protein immunochemistry. While our rps16 intron study suggested that genus *Angelica* (*A. ampla*, *A. pinnata*, *A. breweri*) is included in the *Heracleum* clade (the result is not shown here). The separate analysis of ITS and combined analysis of between ITS and rps16 do not include *Angelica* within the *Heracleum* clade. Close Drude's treatment (1897-98) recognized three subtribes within Peucedaneae on the basis of morphology of the wings. *Angelica* was included within Angelicinae characterized by lateral wings which separate during maturation. However, *Pastinaca* and *Heracleum* are included in Ferulinae (Peucedaninae) and Tordyliinae respectively and characterized by closely appressed lateral wings and thickened wing margins. *Conium maculatum*, *Apium graveolens*, *Ammi majus*, *Anethum graveolens*, and *Angelica* species are used for outgroup. All present separate and combined studies (except rps16 intron) support their sister status or outgroup to the major *Heracleum* clade. A very recently published study of SW Asian and European Apiaceae includes further sampling from this clade and agrees with these results with the additional inclusion of European species of *Heracleum*, *Semenovia*, and *Ainsworthia* within the expanded '*Heracleum* Clade' which is now equated with Tribe Tordylieae (Yousef et al., In press).

Polyphyly of Sino-Himalayan *Heracleum*

The results from the combined and separate analyses have consistently revealed that the genus *Heracleum* is not monophyletic, but polyphyletic because *Heracleum* species are distributed throughout the major *Heracleum* clade and several genera (*Pastinaca*, *Malabaila*, *Semenovia*, *Zosima*, *Tordyliopsis* and *Tordylium*) are nested within *Heracleum sensu lato*. Two main lineages have been identified within Sino-Himalayan *Heracleum* (Fig 1, 2).

Heracleum group I (Fig. 1) is well supported and characterized by highly reduced calyx teeth (absent or poorly developed), broad and inconspicuous dorsal ribs, distinctive clavate vittae which are more or less half length of mericarp. We have divided this clade into three sub-clades (A1, A2, A3) which also correspond to geographical distribution.

Sub-clade A1 contains *H. franchetii*, *H. scabridum, H. stenopterum* and *H. forrestii* species which are exclusively from SW and NW China. This clade is strongly supported (87% bootstrap value). Within this clade the samples of *H. franchetii* and *H. scabridum* form well supported groups and appear to be good species, although the similarity of vegetative characters across this sub-clade makes identification very difficult as the species boundaries are often blurred. Very little differences in sequences were seen between these and the other samples in this clade, and it is notable that the *H. stenopterum* and *H. forrestii complex* species have identical or only 1-2 base pair different changes. More extensive sampling in China will be needed to resolve the taxonomic problems in this group.

Sub-clade A2 is weakly supported, but forms a distinctive clade which is mainly composed of species allied to *Heracleum sphondylium* from NE China including Korea and Japan, through to North America (*H. lanatum* 2). Within this sub-clade only species from Korea (*H. moellendorfii*) and from Japan (*H. lanatum* 1) form a well supported group. The past identification of Japanese *Heracleum* taxa as *H. lanatum* (1) appears dubious because not only is this taxon morphologically very similar to *H. moellendorfii* from Korean, but it is now seen to be genetically very close with only two bp differences. NE Chinese taxa (*H.tiliifolium, H. moellendorfii*) and one North American taxon (*H. lanatum* 2) are not resolved in our analyses. *H. lanatum* is known only one separate distribution on North America. This is commonly accepted typical east Asian, eastern North American disjunction (Wen, 1999). In Apiaceae several examples of this distribution patterns, *Osmorhiza* (Wen, J. & al. 2001), *Crytotaenia* (Li, 1952) are reported. In the ITS analysis the *H. mantegazzianum* clade arises inside clade A2 (Fig. 1) and sister to the *H. moellendorfii* clade. But in the BA analysis this clade is isolated and sister to all other A1 species.
Sub-clade A3 consists of *H. sphondylium* and *H. dissectum* as sister to all clades A1 and A2. These taxa have identical sequences even though they were collected from widely different areas. *H. sphondylium* from UK and *H. disectum* from Central Siberia (Russia) forms well supported clade B3. Although *H. dissectum* is often distinguished from *H. sphondylium* on the basis of overall size and degree of leaf dissection, many people identify them on chorology alone.

The taxonomy of the *Heracleum sphondylium* complex is highly problematic with many species described in the past on poor characters of differences in leaf segmentation, lobe and tooth architecture. More recently taxonomic treatments of European taxa now treat *H. sphondylium* as a morphologically very variable species. Such a broad species concept of *Heracleum sphondylium* has been followed by Briquet (1924), (Thellung, 1925-1926), and Brummitt (1972), and in North American *H. lanatum* has been treated in *H. sphondylium* (as subsp. *montanum*) by Brummitt (1972). In Japan this broad specie concept has also been adopted in Flora of Japan (Ohba, 1999) all Japanese taxa are treated under *H. sphondylium*. Our results generally support these treatments, although further study would be needed with broader sampling before sweeping changes can be made to the classification of this complex worldwide.

***Heracleum* Group II**
Heracleum (17 accessions), *Semenovia* (2 species) and the monotypic *Tordyliopsis* compose a distinctive group (*Heracleum* Group II) of plants from the mountains of the Himalayan region. Although this group appears to be a natural assemblage of species, it is, however, poorly supported, with a bootstrap value of less than 50%.

Within this larger group several sub groups can be usefully recognised. Inside sub group B1 *H. wallichii* forms a monophyletic group with its sister species *H. sublineare*: they are closely related and sometimes difficult to separate on morphology alone. However, fruit wing characteristics, such as broadness of the marginal wing are diagnostic, with *H. wallichii* having wings definitely broader than the fruit body.

Heracleum nepalense and *H. woodii* comprise a monophyletic group and sister to *H. kingdonii*. *Heracleum nepalense* is morphologically a rather variable species widely distributed across the Himalaya and into SW China, and it is often misidentified in herbaria. *Heracleum woodii* was described by Watson (1999) in flora of Bhutan and separated from *H. nepalense* using number of characters (yellowish green flowers, small number of rays, solitary

subtending lateral umbel), but many of the fruit morphological characters such as wing margins and dorsal ribs characters are very similar to *H. nepalense.* In the past *H. woodii* has been treated as *H. nepalense* D. Don var. *bivittatum* Clarke, and our ITS and ITS, rps16 combined analyses also shows their close relationships. Wider sampling of *H. nepalense* would be desirable to confirm this relationship and to indicate the most appropriate rank for the Bhutanese plant. But *H. nepalense* has proven difficult to extract good quality DNA and of the many accessions we used only one produced usable sequences.

Heracleum hookerianum and *Tetrataenium rigens* (Wall. ex DC.) Manden. (=*H. rigens* Wall.) are restricted to the southern part of India (Nilgiri hills, Peninsular India) and are not resolved, but cluster with the Himalayan *H. bhutanicum* and a new species of *Heracleum*. They share yellow or yellowish-white flowers and terminal and lateral peduncles. Because of its flower colour and inflorescence, several south Indian *Heracleum* taxa (e.g. *Heracleum sprengeleanum* Wight & Arn, *H. candolleanum* (Wight & Arn.) Gamble, etc.) have in the past been included in the genus *Pastinaca* (Wight and Arnott, 1834). Our analyses confirm that at least two of these South Indian *Heracleum* taxa should be included in *Heracleum* rather than *Pastinaca*. The *Heracleum bhutanicum* clade has good bootstrap support, but the relationships with other nearby species are not clear. *H. bhutanicum* is endemic to Bhutan and this is low altitude, yellow flowered, hairy species only known from Chukka district SW Bhutan. The new species of *Heracleum* "*H. new species"* was very recently collected by the senior author from Chukai in NW Yunnan, China. This is the subject of an ongoing investigation and will be describe later. Preliminary study of fruit anatomy shows that it is different from all known *Heracleum* species.

Heracleum candicans and *H. obtusifolium* with *H. pinatum* and *H. canescens* form a group (B2) which is moderately well supported. However, within this group *H. candicans* and *H. obtusifolium* always form a strongly supported monophyletic group in all analyses. These two species are very variable, particularly in the size and dissection of the leaves and the shape of the leaflets, and the distinction between two species has long been problematic. Cannon (1978) commented that *H. obtusifolium* is probably only a minor variant of *H. candicans*, and suggested that further investigation is need to confirm its status. Pu & Watson (2005) treated *H. obtusifolium* as variety of *H. candicans* (*H. candicans* var. *obtusifolium* (Wallich ex DC.) F. T. Pu & M. F. Watson), and our phylogeny confirms that these two closely related taxa are difficult to separate this shows that two taxa are very closely related are more appropriately treated as conspecific.

Tordyliopsis brunonis has been treated as a *Heracleum brunonis* by past authors (Pimenov & al., 2000). Our results show that this species is rather distinct from most Himalayan *Heracleum* species, but it is closely related and the separation of *Tordyliopsis* from *Heracleum* purely on our molecular evidence is not strongly supported. De Candolle separated this genus first time on the basis of the distinctive involucre of several narrow, tomentose, mostly subentire bracts about half as long or longer than the rays, and involucel of conspicuous bracteoles similar to the bracts. In our micro-morphological examination using SEM we found that *Tordyliopsis brunonis* has unique glandular trichomes not seen in the other *Heracleum* species (Paik & Watson, unpublished data). On the basis of combined evidence we uphold the treatment of this distinctive species in a separate monospecific genus.

H. cachemiricum shows always closely related with two species of *Semenovia*. Alava (1987b) once transferred this species to *Tricholaser* Gilli, as *T. cachemericum* (C. B. Clarke) Alava,

comb. nov. on the basis of the hermaphrodite and male flowers in the same umbellet, the slightly tumescent fruit wing margins, and subequal dorsal vittae extending nearly to the fruit base. However, this change was not adopted by recent floristic account in Himalaya regions (Mukherjee & Constance, 1993; Nasir, 1971; Watson, 1999).

Semenovia is mostly a central Asiatic genus (SW, S, Central Asia) comprising around 18 species, sometimes included within and sometimes separated from *Heracleum* (Pu & Watson, 2005; Watson, 1999). This genus circumscribed by linear dorsal vittae which run the full length of the mericarp, fruit wing margins that are not inflated, and an unsclerified inner mesocarp layer (Alava, 1987a; Pu & Watson, 2005). However, the delimitation of this genus has been trouble. In their treatment of the Afghanistan representatives of the genus (*S. lasiocarpa* complex), Rechinger & Rieldl (1963) stated that "the taxon is a very polymorphic one so the treatment is considered provisional." In "Flora Iranica" Alava (1987a) also suggested the necessity of more compressive survey of the genus because many of the these species are known only from often scanty and fragmentary type collection as well as many of them are lack of characters of diagnostic value (e.g. mature fruit, flowers , basal leaves). In our analyses two species of *Semenovia* always appears within the *Heracleum* Group II. Results from a very recent phylogenetic study of Iranian Apiaceae using ITS sequences (Yousef & al. In press) also supported our results. In this study two *Semenovia* taxa (*S. dichotoma*, *S. fragioides*) are nested inside their *Heracleum* clade and are placed close to the *H. candicans* clade. Clearly, further work on this enigmatic genus is needed, with more extensive sampling beyond the four species included in these two studies, before any taxonomic changes can be proposed.

Apart from two *Semenovia* species many species within our *Heracleum* Group II correspond to Mandenova's (1959, 1982) genus *Tetrataenium*. This generic circumscription is further supported by fruit morphological characters: well-developed calyx teeth; prominent and narrowly carinate dorsal and intermediate fruit ribs; and elongate, truly claviform and septate dorsal vittae. However, adopting a broader circumscription of this genus to cover all elements in our *Heracleum* Group II would be very far reaching, and premature. Before this, further studies on the morphological and anatomical characters of these species is need together with more exhaustive sampling for molecular analysis, especially of *Semenovia*.

Relationships between *Heracleum* and its sister genera

***Pastinaca-Malabaila* clade** (Fig. 1, 2) includes *Pastinaca* (cultivated worldwide) and *Malabaila* (Mediterranean) in a group with a strong support (bootstrap 99%) and characterized by yellow petals, and simply pinnate leaves. It is interesting to note the placement of this clade close to the core *Heracleum* clade (*Heracleum* Group I), a result also supported by other recent studies (Yousef & al. In Press; Logacheva & al., In Press). Calestani's (1905) rather radical re-classification of these genera included *Malabaila*, *Heracleum*, *Zosima*, *Lophotaenia*, *Ainsworthia*, *Wendiana*, and *Tordylium* within *Pastinaca*. Later studies refuted this classification, and Menemen & Jury (2001a, b, c) suggested that these genera could be clearly separated from each other using characteristics of macro-morphology, micromorpholgy, anatomy, and palynology. Our molecular analyses also support treating these genera as distinct, but in our results the taxonomic relationships between species of *Pastinaca* and *Malabaila* are still ambiguous. In our results the placement of *Malabaila* is still unresolved as its type species is closely related to *Pastinaca* s. str., but the remaining species are dissimilar in fruit characters (Paik & Watson, unpublished data).

proposed remove these remaining species and treating them in the neglected genus *Leiootulus* Ehrenb. However, later detailed morphological analyses by Menemen &Jury (2001b) showed no nomenclatural changes were necessary.

The *Zosima* clade is strongly supported with a bootstrap value of 99% (Fig.1, Fig. 2). This distinctive genus is characterised by its large vittae, which completely fill the space between the two ribs, the linear leaf segments (Menemen & Jury, 2001c), and the development of distinctive parenchyma cells in the proximal regions (Paik & Watson, unpublished data). It also has a distinctive composition of flavonols and flavones (Menemen & al. 1998), and in all our analysis it clearly forms a monophyletic group. The relationship of this group to the other clades is less clear as it forms a polytomy almost at the base of the ITS phylogeny and appears in a polytomy rather higher up the tree in the ITS and rps16 intron combined analysis. Further sampling with *Zosima* and using other gene sequences is needed resolve these issues.

Taxonomic placement of some enigmatic taxa

The taxonomic position of *Heracleum millefolium* has long been problematic. In the Flora of Bhutan (Watson, 1999) this taxon was treated within *Semenovia* (*S. millefolia*) based on the dwarf perennial habit with a tough fibrous stem base, mostly basal pinnate leaves and fruit with dense white hairs. However, in the Flora of China (Pu & Watson 2005) this species was placed within the genus *Heracleum* on account of the radiate outer flowers and clavate vittate. Our result shows that the taxonomic position of *H. millefolium* is still uncertain, but at least its separation from all *Heracleum* species sampled (and the other allied genera sampled including *Semenovia*) is supported. Further sampling of *Semenovia* and related genera will help to confirm its position as at the moment it does not sit well in either *Heracleum* nor *Semenovia*, our results suggesting the description of a new genus for this enigmatic species.

The taxonomic position of *Tordylium* has been contentious because previous classifications were wholly based on suits of morphological characters. El-Eisawi & Jury (1988) revised the genus *Tordylium* and four related genera: *Ainsworthia* Boss., *Tordylium* L., *Synelcosciandium* Boiss., and *Mandenovia* Alava. In this revision *Ainsworthia* and *Synelcosciadium* were treated within *Tordylium*, and *Mandenovia* as a monotypic genus. The genus *Tordylium* was divided into subgenus *Tordylium* (including *Synelcosciadium*) and subgenus *Ainsworthia* (Boiss.) Drude. In our phylogenetic analysis we included two species of *Tordylium* (*T. apulum* and *T. elegans*), which do not cluster together and are widely placed in all our trees. El-Eisawi & Jury (1988) placed both of these species in subgenus Ainsworthia, but *T. apulum* within section *Condylocarpus* (Hoffm.) DC. and *T. elegans* within section *Hasselquist*ia (L.) Boiss. In other recent studies *T. aegyptiacum* var. *palaestinum* is also oddly placed (Yousef & al*.,* In press)*,* and *T. maximum* (the type species of the genus) is placed sister to the *Pastinaca* and *Heracleum* group (Locheva & al. In press). Our results and these recent studies indicate that a detailed molecular study of *Tordylium sensu lato* would be a very important data source for resolving these problems.

Conclusion

In order to evaluate the monophyly of Sino-Himalayan *Heracleum*, and clarify the species relationships within this group, additional sequences of ITS, rps16 intron of *Heracleum* and related genera were analysed. Both ITS and rps16 intron regions show a good resolution of phylogenetic relationships down to species level. Our comparative analysis refined monophyly of "*Heracleum* clade" sensu Downie. Within this clade several major lineages are defined: *Heracleum*, *Malabaila-Pastinaca*, *Zosima*, *Semenovia*, *Tordylium*, and *Tordyliopsis*. The Sino-Himalayan *Heracleum* lineage is seen to comprise two distinct sub-lineages

(*Heracleum* Groups I & II) including monotypic genus *Tordyliopsis* and Central Asiatic genus *Semenovia*. *Heracleum* is thus not monophyletic. Generic segregates of *Heracleum* proposed in past classifications, such as *Tetrataenium,* can be correlated with Heracleum Group II. These phylogenies also helped resolve the taxonomic placement of some enigmatic taxa such as *Heracleum millefolium* and *Tordylium.*

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APPENDIX

List of accessions used in the phylogenetic analysis of nrDNA ITS and cpDNA rps16 intron sequence data. Previously published ITS and rps16 intron sequences are indicated with GenBank reference numbers. For the newly obtained sequences, voucher information is provided along with the GenBank reference number. GeneBank reference numbers for newly obtained ITS sequences include ITS1, ITS2 and the intervening 5.8S data.

Previously published Sequence from GeneBank
ITS sequence data: *Ammi majus* L., Downie et al. 1998, U78386 &U78446; *Anethum graveolens* L., Downie and Katz-Downie 1996, AH003470; *Angelica ampla* A. Nelson, Downie et al. 1998, AH006065; *Angelica breweri* A. Gray, Downie et al. 1998, U78396 & U78456; *Angelica pinnata* S. Watson, Downie et al. 2002, AF358465; *Apium graveolens* L., Downie and Katz-Downie 1996, AH003471; *Conium maculatum* L., Downie et al. 1998, AH006070; *Heracleum lanatum* Michx., Downie et al. 1996, AH003476; *Heracleum moellendorfii* Hance 2, Choi, et al. 2004, AY548226; *Malabaila secacul* (Banks & Sol.) Boiss., Katz-Downie et al. 1999, AF008627; *Pastinaca armena* Fisch. & Mey., Katz-Downie et al. 1999, AF008626; *Pastinaca sativa* L., Downie et al. 1996, AH003484; *Tetrataenium rigens* (Wall. ex DC.) I.P. Mandenova, Downie et al. 1996, AH003477; *Zosima orientalis* Hoffm., Katz-Downie et al. 1999, AF008628; **rps16 intron sequence data:** *Ammi majus* L. Downie et al. 2000, AF164814; *Anethum graveolens* L., Downie and Katz-Downie 1999, AF110542; *Angelica ampla* S. Watson, Downie et al. 2002, AF358598; *Angelica breweri* A. Gray, Downie et al. 2002, AF358599; *Angelica pinnata* S. Watson, Downie et al. 2002, AF358600; *Apium graveolens* L., Downie and Katz-Downie 1999, AF110545; *Conium maculatum* L., Downie and Katz-Downie 1999, AF110546; *Heracleum lanatum* Michx. Downie and Katz-Downie 1999, AF110537; *Malabaila secacul* (Banks & Sol.) Boiss., Downie et al. 2000, AF164802; *Pastinaca armena* Fisch. & Mey., Downie et al. 2000, AF164803; *Pastinaca sativa* L., Downie and Katz-Downie 1999, AF110538; *Zosima orientalis* Hoffm., Downie et al. 2000, AF164806; *Tetrataenium rigens* (Wall. ex DC.) I.P. Mandenova, Downie and Katz-Downie 2000, AF164804.

List of taxa analyzed, with vouchers and GenBank accession numbers.
ITS new sequence data: *Heracleum austriacum* L.,Austria, Salzburg 1400-1500m, *M. Eysn w/n* (E), EU185657; *Heracleum bhutanicum* M.F.Watson, Bhutan, Chukka District, Chuka, 1400m, *Watson 6811* (E), EU185652; *Heracleum bhutanicum* M.F.Watson, Bhutan, Chukka District, Chuka, 1500m, *Wood 7158* (E), EU185676; *Tordyliopsis brunonis* Benth. ex C.B.Clarke, Bhutan, Thimphu District, Talukah Gompa, 4200m, *Wood 6616* (E), EU185642; *Heracleum cachemericum* Clarke, Pakistan, Punjab, Charihan Murree Hill 6000fit, *Coventry w/n* (K), EU185649; *Heracleum candicans* Wall. ex DC., China, Yunnan, *Ace 1576* (E), EU185643; *Heracleum canescens* Lindl., Nepal, Jumla, 7,600ft., *Polunin et al 4461* (E), EU185673; *Heracleum dissectum* Ledeb., Central Siberia, ca. 122km SE of Novosibirsk, Alt. 300-400 m, *H. H. Iltis & al. 1224* (NY), EU185691; *Heracleum forrestii* H. Wollf 1, China, Yunnan, Zhongdian County, Wu Feng Shan, *FED 34* (E), EU185684; *Heracleum forestii* H. Wolff 2, China, Yunnan, Gongshan county, Gongshan to Kongdan, 3400m, *GSBS 16833* (E), EU185685; *Heracleum forrestii* H. Wolff 3, China, Yunnan, Chungtien-plateau, 9000 fit, *Forrest 13115* (E), EU185687; *Heracleum forestii* H. Wolff 4, China, Yunnan, Zhongdian county, DaxueShan, 4100m, *FED 199* (E), EU185688; *Heracleum forestii* H. Wolff 5, China, Yunnan, Gaoligongshan, *GSBS 26517* (E), EU185689; *Heracleum forrestii* H. Wolff 6, China, Yunnan, Gaoligongshan, Chukai, *GSBS 31701* (E), EU185660; *Heracleum franchetii* Hiroe, China, Qinghai, Nangqên Xian, *Ho & al. 1752* (BM), EU185661; *Heracleum franchetti* Hiroe, China, Qinghai, Chindu Xian, 3600 m, *Boufford & al. 26967* (E), EU185690; *Heracleum*

hookerianum Wight & Arn., India, Madras, 7000 fit, *Gamble 16960* (K), EU185653; *Heracleum kingdonii* H. Wolff, North Burma, Kachin State, The triangle, 4000 fit, *Kingdon-ward 21305* (BM), EU185675; *Heracleum lanatum* Michx., Japan, Kyoto, *Murata & Koyama 186* (E), EU185656; *Heracleum mantegazzianum* Sommier & Levier 1, United Kingdom, Middle Lothian, *Drever 2* (E), EU185658; *Heracleum mantegazzianum* Sommier & Levier 2, Uinted Kingdom, Middle Lothian, Edinburgh, *Mandenova w/n* (E), EU185662; *Heracleum millefolium* Diels, China, Qinghai, Yushu Xian, 3850-3950 m, *Ho & al. 2087* (E), EU185655; *Heracleum millefolium* Diels, China, Qinghai, Madoi Xian, 4070 m, *Ho & al. 1565* (BM), EU185670; *Heracleum moellendorffii* Hance 1, China, Anhoui, 1200 m, *Zhongwen & Lin 97097* (MO), EU185681; *Heracleum moellendorfii* Hance 3, Korea, Kangwon province, *Lee 0013861* (KWNU), EU185682; *Heracleum moellendorfii* Hance 4, Korea, Kangwon province, *Lee 0053777* (KWNU), EU185683; *Heracleum nepalense* D. Don, Bhutan, Upper Mochu district, 3850 m, *Sinclair & al. 5342* (E), EU185651; *Heracleum obtusifolium* Wall. ex DC., Bhutan, Thimphu, *Wood 7034* (E), EU185644; *Heracleum obtusifolium* Wall. ex DC., China, Qinghai, Yushu Xian, 3600 m, *Ho & al. 2339* (BM), EU185671; *Heracleum pinnatum* C.B.Clarke, India, Padum, Zanskar district, Jammu & Kashmir State, *Grace & al. 3* (K), EU185672; *Heracleum scabridum* Franch., China, Yunnan, *Forrest 2260* (E), EU185667; *Heracleum scabridum* Franch., China, Yunnan, Yangtze watershed, Prefectural District of Likiang, *Rock 5631* (E), EU185686; *Heracleum spp*. (new), China, Yunnan, Gaoligongshan, Chukai, *GSBS 31616* (E), EU185650; *Heracleum sphondylium* L., United Kingdom, Whalsay, *Beanell 13/7* (E), EU185663; *Heracleum stenopterum* Diels, China, Yunnan, 10-11000ft, *Forrest 2847 (*E), EU185659; *Heracleum sublineare* C.B.Clarke, Bhutan, Sankosh District, 3600m, *Wood 7058* (E), EU185654; *Heracleum sublineare* C.B.Clarke, Nepal, Bheding, 12000-13000ft, *Dhwoj 272* (E), EU185677; *Heracleum tillifolium* H.Wolff, China, Hupei, Yang Long co., Long Kan village, *Ye Cunsu 9603* (MO), EU185680; *Heracleum wallichii* DC., Nepal, Malemchi, 7500ft, *Stainton 6666* (BM), EU185678; *Heracleum woodii* M.F. Watson, Bhutan, Thimphu District, Phajoding on ridge above 3900m, *Wood 7138* (E, sheet 3); EU185674; *Malabaila aucheri* Boiss., Iran, D5(Times Atlas): 10km E. of Bebehan, 400m, *Davis & Bokhari 55816* (E), EU185665; *Malabaila involucrata* Boiss. & Spruner, Greece, Roadside at 600 m, *Cghanson w/n* (E), EU185669; *Pastinaca sativa* L., Bulgaria, Sofia, Rila Planina, Musala, 1250m, *Gardner & Gardner 3202* (E), EU185666; *Pastinaca sativa* L., USA, North Carolina, Ashe County, *Pence 44875* (E), EU185668; *Semenovia dasycarpa* Korov. ex Pimenov & V.N.Tikhom., SE Afghanistan, Orozgan: Inter Tirin, 2000m, *K. H. Rechinger 35159* (G), EU185645; *Semenovia transiliensis* Regel & Herd., Kazachstania, declivitas borealis jugi Kungei-Alatau, in fauce fl. Kaindy, in pratulis silvaticis, 2000 m, *I. Roldugin w/n* (NY), EU185646; *Tordylium apulum* L., Turkey, B1 Izmri, cesme 10-50 m, *Davis 41835* (E), EU185679; *Tordylium elegans* (Boiss. & Balansa) Alava & Hub.-Mor. Turkey, Adana, *Alava 6694* (E), EU185664; *Zosima absinthifolia* DC., Azerbaijan, between Khiyav and Ahar, 1100m, *Lamond 3327* (E), EU185647; *Zosima radians* Boiss. & Hohen., Iran, Tehran, Between Tehran and Karadj, 1500 -1900 m, *Assadi & Mozaffarian 27560* (E), EU185648.
rps16 intron new sequence data: *Heracleum austriacum* L., Austria, Salzburg 1400-1500m, *M. Eysn w/n* (E), EU185707; *Heracleum bhutanicum* M. F. Watson, Bhutan, Chukka District, Chuka, 1400m, *Watson 6811* (E), EU185702; *Heracleum brunonis* Benth. ex C.B.Clarke, Bhutan, Thimphu District, Talukah Gompa, 4200m, *Wood 6616* (E), EU185692; *Heracleum cachemericum* Clarke, Pakistan, Punjab, Charihan Murree Hill 6000fit, *Coventry w/n* (K), EU185699; *Heracleum candicans* Wall. ex DC., China, Yunnan, *Ace 1576* (E), EU185693; *Heracleum forrestii* H. Wolff 6, China, Yunnan, Gaoligongshan, Chukai, *GSBS 31701* (E), EU185710; *Heracleum franchetii* Hiroe, China, Qinghai, Nangqên Xian, *Ho & al. 1752* (BM), EU185711; *Heracleum hookerianum* Wight & Arn., India, Madras, 7000 fit, *Gamble 16960* (K), EU185703; *Heracleum lanatum* Michx., Japan, Kyoto, *Murata & Koyama 186* (E),

EU185706; *Heracleum mantegazzianum* Sommier & Levier 1, United Kingdom, Middle Lothian, *Drever 2* (E), EU185708; *Heracleum mantegazzianum* Sommier & Levier 2, United Kingdom, Middle Lothian, Edinburgh, *Mandenova w/n* (E), EU185712; *Heracleum millefolium* Diels, China, Qinghai, Yushu Xian, 3850-3950 m, *Ho & al. 2087* (E), EU185705; *Heracleum nepalense* D. Don, Bhutan, Upper Mochu district, 3850 m, *Sinclair & al. 5342* (E), EU185701; *Heracleum obtusifolium* Wall. ex DC., Bhutan, Thimphu, *Wood 7034* (E), EU185694; *Heracleum scabridum* Franch., China, Yunnan, *Forrest 2260* (E), EU185721; *Heracleum spp*. (new), China, Yunnan, Gaoligongshan, Chukai, *GSBS 31616* (E), EU185700; *Heracleum sphondylium* L., United Kingdom, Whalsay, *Beanell 13/7* (E), EU185713; *Heracleum stenopterum* Diels, China, Yunnan, 10-11000ft, *Forrest 2847*(E), EU185709; *Heracleum sublineare* C.B.Clarke, Bhutan, Sankosh District, 3600m, *Wood 7058* (E), EU185704; *Malabaila aucheri* Boiss., Iran, D5(Times Atlas): 10km E. of Bebehan, 400m, *Davis & Bokhari 55816* (E), EU185716; *Pastinaca sativa* L., Bulgaria, Sofia, Rila Planina, Musala, 1250m, *Gardner & Gardner 3202* (E), EU185717; *Semenovia dasycarpa* Korov. ex Pimenov & V.N.Tikhom., SE Afghanistan, Orozgan: Inter Tirin, 2000m, *K. H. Rechinger 35159* (G), EU185695; *Semenovia transiliensis* Regel & Herd., Kazachstania, declivitas borealis jugi Kungei-Alatau, in fauce fl. Kaindy, in pratulis silvaticis, 2000m, *I. Roldugin w/n* (NY), EU185696; *Tordylium elegans* (Boiss. & Balansa) Alava & Hub.-Mor., Turkey, Adana, *Alava 6694* (E), EU185714; *Zosima absinthifolia* DC., Azerbaijan,between khiyav & Ahar, 1100m, *Lamond 3327* (E), EU185697; *Zosima radians* Boiss. & Hohen., Iran, Tehran, Between Tehran and Karadj, 1500 -1900m, *Assadi & Mozaffarian 27560* (E), EU185698.

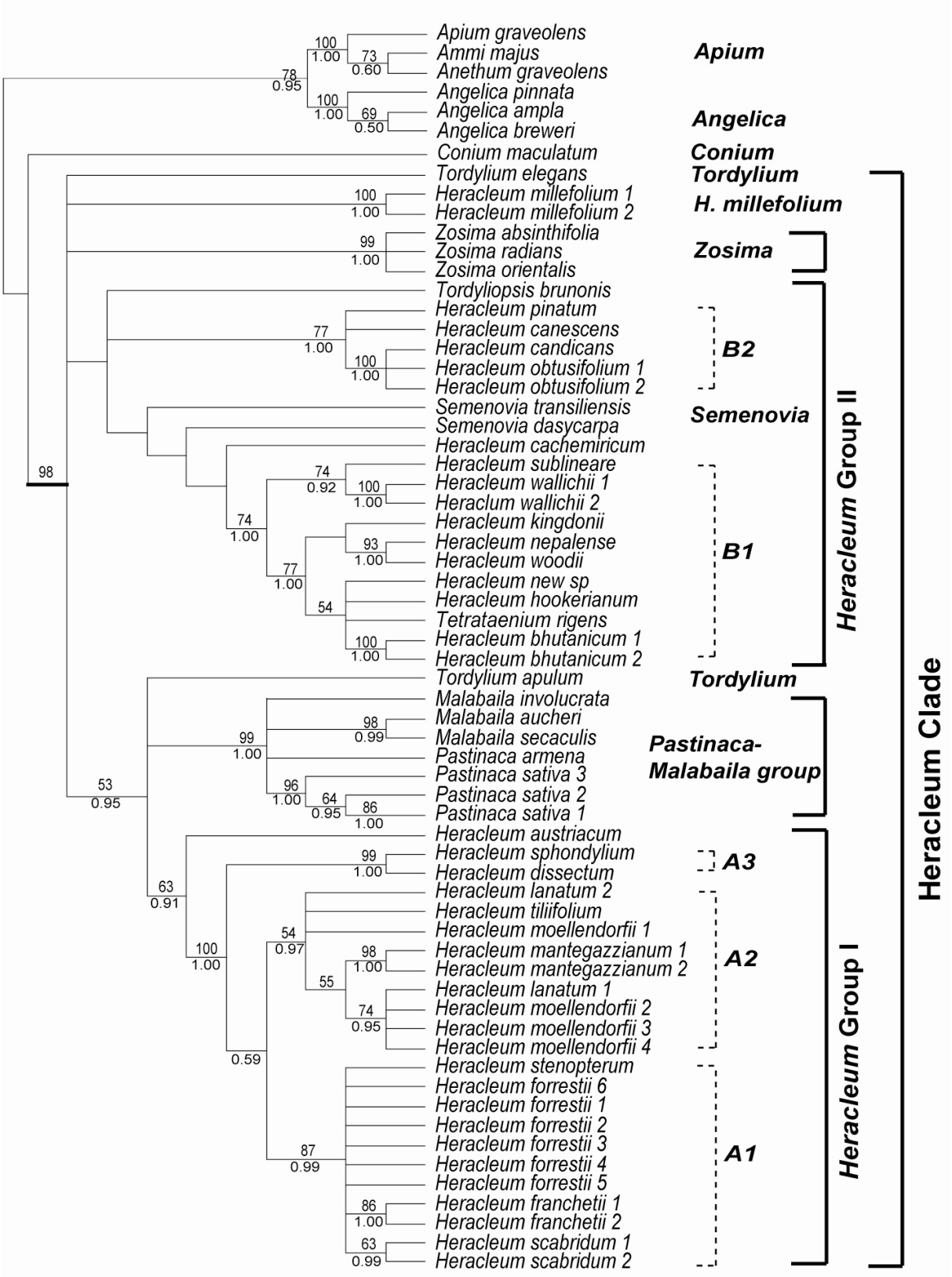
LEGENDS

Table 1.
Sequence characteristics of ITS and rps16 intron regions, separately and combined, used in the phylogenetic analysis of *Heracleum* and related genera. *Based on alignment excluding ambiguous sequence sites.

Table 2.
The result of the parsimony analysis. All characters except ambiguous sequences; Consistency Indices (CI) based on informative characters only; ( ): number of taxa.

Figure 1.
Strict consensus tree of 128 most parsimonious trees, each 584-steps long, derived from equally weighted maximum parsimony analysis of 64 nuclear nrDNA ITS sequences from Sino-Himalayan *Heracleum* and related genera with outgroups (CI excluding uninformative characters = 0.376; RI = 0.748). Bootstrap values above 50% are indicated at the respective nodes. Brackets indicate clade descriptors discussed in the text.

Figure 2.
Bayesian majority rule consensus tree obtained from 39 accessions of the combined ITS and rps16 intron sequences from Sino-Himalayan *Heracleum* and related genera with the inclusion of gap characters. Numbers above braches are estimated posterior probability values.


Apium graveolens
Ammi majus
Anethum graveolens
Angelica pinnata
Angelica ampla
Angelica breweri
Conium maculatum
Tordylium elegans
Heracleum millefolium 1
Heracleum millefolium 2
Zosima absinthifolia
Zosima radians
Zosima orientalis
Tordyliopsis brunonis
Heracleum pinatum
Heracleum canescens
Heracleum candicans
Heracleum obtusifolium 1
Heracleum obtusifolium 2
Semenovia transiliensis
Semenovia dasycarpa
Heracleum cachemiricum
Heracleum sublineare
Heracleum wallichii 1
Heraclum wallichii 2
Heracleum kingdonii
Heracleum nepalense
Heracleum woodii
Heracleum new sp
Heracleum hookerianum
Tetrataenium rigens
Heracleum bhutanicum 1
Heracleum bhutanicum 2
Tordylium apulum
Malabaila involucrata
Malabaila aucheri
Malabaila secaculis
Pastinaca armena
Pastinaca sativa 3
Pastinaca sativa 2
Pastinaca sativa 1
Heracleum austriacum
Heracleum sphondylium
Heracleum dissectum
Heracleum lanatum 2
Heracleum tiliifolium
Heracleum moellendorfii 1
Heracleum mantegazzianum 1
Heracleum mantegazzianum 2
Heracleum lanatum 1
Heracleum moellendorfii 2
Heracleum moellendorfii 3
Heracleum moellendorfii 4
Heracleum stenopterum
Heracleum forrestii 6
Heracleum forrestii 1
Heracleum forrestii 2
Heracleum forrestii 3
Heracleum forrestii 4
Heracleum forrestii 5
Heracleum franchetii 1
Heracleum franchetii 2
Heracleum scabridum 1
Heracleum scabridum 2
Apium
Angelica
Conium
Tordylium
H. millefolium
Zosima
B2
Semenovia
B1
Tordylium
Pastinaca-
Malabaila group
A3
A2
A1
Heracleum Group II
Heracleum Group I
Heracleum Clade

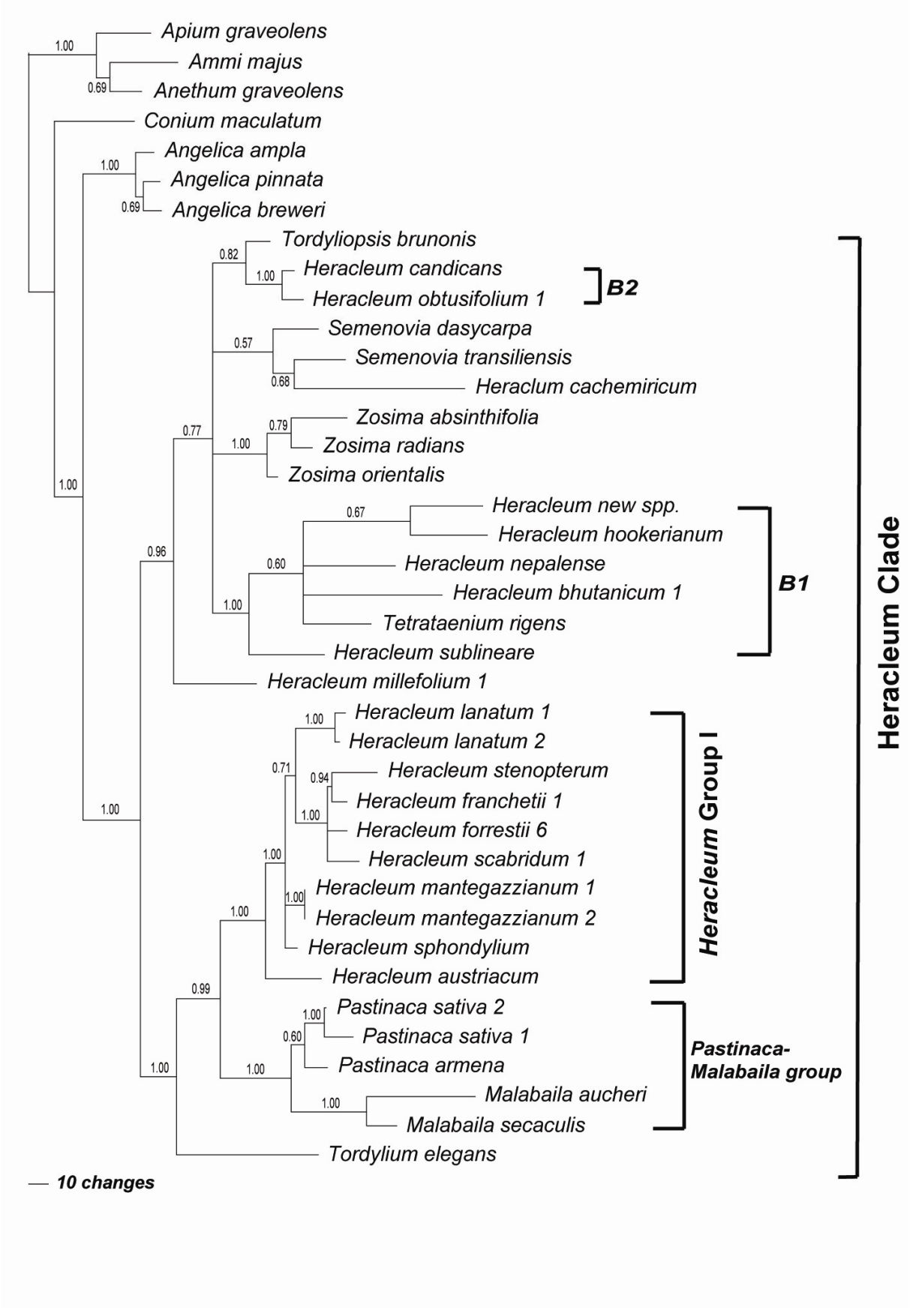

Apium graveolens
Ammi majus
Anethum graveolens
Conium maculatum
Angelica ampla
Angelica pinnata
Angelica breweri
Tordyliopsis brunonis
Heracleum candicans
Heracleum obtusifolium 1
B2
Semenovia dasycarpa
Semenovia transiliensis
Heraclum cachemiricum
Zosima absinthifolia
Zosima radians
Zosima orientalis
Heracleum new spp.
Heracleum hookerianum
Heracleum nepalense
Heracleum bhutanicum 1
Tetrataenium rigens
Heracleum sublineare
B1
Heracleum millefolium 1
Heracleum lanatum 1
Heracleum lanatum 2
Heracleum stenopterum
Heracleum franchetii 1
Heracleum forrestii 6
Heracleum scabridum 1
Heracleum mantegazzianum 1
Heracleum mantegazzianum 2
Heracleum sphondylium
Heracleum austriacum
Heracleum Group I
Pastinaca sativa 2
Pastinaca sativa 1
Pastinaca armena
Malabaila aucheri
Malabaila secaculis
Pastinaca-Malabaila group
Tordylium elegans
Heracleum Clade
10 changes

Table 1. Sequence characteristics of ITS and rps16 intron regions,separately and combined, used in the phylogenetic analysis of *Heracleum* and related genera. *Based on alignment excluding ambiguous sequence sites

Parameter	ITS	rps16	Combined ITS+rps16
Length range (total) (bp)	420-443	783-878	1203-1321
Length mean (total) (bp)	436.92	853.74	1290.66
Aligned length (bp)	616	966	1582
Number of excluded site	197	93	290
Number of parsimony informative indels (ingroup)*	3	9	12
Number of parsimony informative indels (total)*	6	16	22
Size of indels (ingroup)*	2-3	5-22	2-22
Size of indels (total)*	2-3	5-22	2-22
Number of sites after exclusion*	419	878	1297
Number of variable sites*	253	113	366
Number of constant sites *	166	765	931
Number of informative sites*	163	56	219
Number of autapomorphic sites *	90	57	147
Sequence divergence (ingroup) *	0-18.0	0-2.85	0-18.0
Sequecne divergence (in/outgroup)*	11.3-24.3	1.03-4.49	1.03-24.3

Table 2. The result of the parsimony analysis. All characters except ambiguous sequences; *:Consistency indices (CI) based on informative characters only; ( ): number of taxa.

	No. of characters in analysis	No. of best trees	Length of best tree	Consistency index (CI)	Retention index (RI)	Rescaled consistency index (RC)
ITS (64)	419	128	584	0.611/0.521*	0.793	0.484
rps16 intron (39)	878	6	151	0.781/0.467*	0.851	0.665
ITS + rps16 (39)	1310	343	667	0.663/0.484*	0.764	0.506

Appendix 3 Floristic account of Nepalese *Heracleum*

→ According to my result all Nepalese Heracleum species move into genus Tetrataenium (see Chapter 8, 9)

Tetrataenium (DC.) Mandenova, Trudy Tbilissi Bot. Inst. 20: 16. 1959.

Heracleum sect. *Tetrataenium* DC., Prodr. 4: 191. 1830.
Tetrataenium (DC.) Mandenova Trudy Tbilis. Bot. Inst., 20: 16 (1959).

Herbs, perennial rarely biennial. Root stout or slender, cylindrical. Stems solitary, stout or weak, erect or ascending, branched, densely or sparsely pubescent. Basal and lower leaves petiolate; petiole sheaths broad or narrow; Leaf blade oblong to broad ovate, ovate to triangular, ternate, pinnate, 1-2 ternate, ternate to pinnate, 1-2 pinnate compound, hairy or glabrous. Umbels loose compound, terminal and lateral, terminal umbel with bisexual flowers, the lateral often with only staminate flowers; bracts few or absent, often caduceus; rays numerous, spreading-ascending; bracteoles several, linear to lanceolate, entire, toothed; umbellules many-flowered. Calyx teeth triangular, lanceolate, lineare or obsolete. Petals white, greenish yellow, pinkish or brownish-yellow; outer flowers of the umbel often radiant with outer petals enlarged, broadly obovate, apex deeply 2-lobed. Fruit elliptic, orbicular, narrowly oblong, obovoid, ovate to obovate, strongly dorsally compressed, hairy or glabrous; dorsal and intermediate (lateral) ribs filiform, sometimes raised, marginal ribs usually winged; vittae 1(-2) in each furrow, 2-4 or absent on commissure. filliform or subclavate, reaching to the base of mericarp or much shorter than the mericarp. Seed face plane, rarely slightly concave. Carpophore parted to base, usually persistent.

Previous classification concept of genus *Tetrataenium* (Sensus Mandenova) is resurrected here. This classification is supported by present study (general fruit morphology, fruit anatomy, and DNA phylogenetic study). This group would certainly include the members of *Semenovia* sampled for this analysis, but further research into the remainder of *Semenovia* (18 species in total) is required to determine if the entire genus should be subsumed within *Tetrataenium*.

About 22 species: maily in Asia (S, SW, Middle); 7 species in Nepal

Key to species

1a. Leaves white tomentose beneath; Cauline leaf sheaths conspicuous.

1. *T. candicans*

2a. leaflets oblong-ovate, apex mucronate or narrowly obtuse; wings equal or slightly narrower than body. **1a. *T. candicans* var. *candican***

2b. leaflets ovate-broad ovate or rotund, apex rounded or broadly obtuse; wings wider than body.

1b. *T. candicans* var. *obtusifolium*

1b. Leaves pubescent but not white tomentose beneath; Cauline leaf sheaths inconspicuous or narrow.

3a. Stems slender; Umbels < 15 cm across.

4a. Stem erect or ascending, densely pubescent on juvenile part; leaflets oblong to ovate.

2. *T. canescens*

4b. Stem erect, moderately pubescent; leaflets linear, linear-lanceolate to narrowly ovate.

5a. Fruit obovoid; wings wider than body **3. *T. wallichii***

5b. Fruit oval to orbicular; wings narrower than body **4. *T. sublinear***

3b. Stems stout; Umbels > 15 cm across.

6a. Fruit narrowly winged; vittae subclavate, extending to base. **5. *T. lallii***

6b. Fruit equally or widely winged; vittae filiform, extending 1/2-2/3 of mericarp.

7a. Main umbels > 20 rays; distinctly overtopped by a pair of subtending secondary lateral umbels; calyx teeth linear. **6. *T. nepalense***

7b. Main umbels < 20 rays; not overtopped by the solitary subtending lateral umbel; calyx teeth triangular. **7. *T. woodii***

1. ***Tetrataenium candicans*** (Wall. ex DC.) Manden. Zametki Sist. Geogr. Rast. 41: 44 (1986).
Heracleum candicans Wallich ex de Candolle, Prodr. 4: 192. 1830.

Plants 40-100(-200) cm high, pubescent or white-tomentose. Root stout, cylindrical. Stem solitary, branched. Basal leaves petiolate, the sheath conspicuously sheathing, leaf blade ovate to oblong, 10-40 x 10-35 cm, pinnate or ternate-pinnate; pinnae 2-4 paris. leaflets oblong to ovate, 5-15 x 3-10 cm, lobed to pinnatifid, abaxially silvery, densely white tomentose, margins serrate or dentate, apex mucronate or obtuse. Cauline leaves reduced, fewer and narrower, 3-lobed on expanded sheaths. Peduncles stout, 8-25 cm, pubescent; bracts 1-3, linear, caducous; rays 15-40, unequal, 3-8 cm, pubescent; bracteols 5-8, linear to lanceolate, umbellules 20-30flowered. Calyx teeth prominent, linear. Petals white, outer flowers of umbels radiant. Fruit orbicular to obovate, 5-8(-10) x 4-6 mm, glabrous when mature; dorsal ribs prominent, filliform, marginal ribs broadly winged, wings narrower to wider than body; vittae large, linear, solitary in each furrow, extending to 2/3 length of mericarp, 2-4 on commissure, linear, extending to 2/3 length of mericarp. Fl. May-Jul., fr. Aug.-Sep.

A widespread species of the Sino-Himalaya, very variable particularly in leaf size, dissection and leaflet shape, two varieties may be recognized.

1a. Leaflets ovate-oblong, apex mucronate or narrowly obtuse, wings equal or slightly narrower than body 1a. ***T. candicans*** var. ***candicans***
1b. Leaflets ovate, broad ovate or rotund, apex rounded or broadly obtuse, wings wider than body 1b. ***T. candicans*** var. ***obtusifolium***

1a. ***Tetrataenium candicans*** **var.** ***candicans***
Heracleum candicans Wallich ex de Candolle, Prodr. 4:191. 1830

Pinnae ovate-oblong, apex mucronate or narrowly obtuse, wings equal or slightly narrower than body

Distribution: West, Central, East Nepal: 2200-3800 m. [N India, Kashmir, Pakistan, Sichuan, E, S Xizang, NW, C, NE Yunnan].

1b. ***Tetrataenium candicans*** **var.** ***obtusifolium*** (Wallich ex de Candolle) Paik & M.F. Watson, stat. nov.
Heracleum obtusifolium Wallich ex de Candolle, Prodr. 4:191. 1830; *Tetrataenium obtusifolium* (Wallich ex de Candolle) Mandenova; *Heracleum candicans* var. *obtusifolium* (Wallich ex de Candolle) F. T. Pu, stat. nov.

Pinnae ovate, broad ovate or rotund, apex rounded or broadly obtuse, wings wider than body

Distribution: West, Central, East Nepal: 2200-3800 m. [N India, Kashmir, Pakistan, Sichuan, E, S Xizang, NW, C, NE Yunnan].

2. ***Tetrataenium canescens*** (Lindl.) Manden., Zametki Sist. Geogr. Rast. 42: 12. 1991. *Heracleum canescens* Lindl., Royle, Illustr. Bot. Himal. Mount. 232. 1835; *Heracleum hirsutum* Edgew., Trans. Linn. Soc. 20: 57. 1851.

Plants 40-80 cm high. Root slender, cylindrical. Stem slender, weak, erect or ascending, branched, densely or finely pubescent on juvenile parts. Basal leaves petiolate; leaf blade oblong to narrowly ovate. 8-15 x 5-10 cm, pinnate, pinnae 2-5 pairs; leaflets oblong to narrowly ovate, 2-5 x 1-2 cm, base cuneate, apex acute or rounded, margins crenate to serrate, pubescent both surface. Cauline leaves similar to the basal, reduced upward, with inconspicuous sheaths. Peduncles slender, 10-35 cm. Umbells 5-10 cm across; bracts 1-3, linear or lacking; rays 7-12, 3-5 cm, unequal, pubescent; bracteols 4-6, lanceolate, margin toothed; umbellules 12-16 flowered. Cayx teeth prominent, linear, or sometimes absent. Petals white, oval to obovate, the outer strongly radiant, unequally emaginate. Fruit oval to obovate, 10-12 x 8-10 mm, sparsely pubescent all over; dorsal ribs filliform, maginal ribs broadly winged, wings equal or slightly narrower than body; vittae large, linear, solitary in each furrow, extending to 2/3 length of mericarp, 2 on commissure, filliform, extending to 1/2 length of mericarp. Seed face plane. Fl. and fr. Aug.-Sep.

Distribution: West Nepal: 3000- 4200 m [Western Himalaya to Pakistan].

3. ***Tetrataenium wallichii*** (DC.) Manden., Zametki Sist. Geogr. Rast. 41: 47 (1986). *Heracleum wallichii* DC**.,** Prodr. (DC.) 4: 195. 1830; *Heracleum diversifolium* Wallich, Cat. 574. 1829, nomen nudum.

Plants 30-80(-120) cm high. Root slender, cylindrical. Stem slender, solitary, branched, pubescent. Basal and lower leaves long petiolate; leaf blade ovate to triangular, 10-20 x 8-15 cm, ternate or biternate; leaflets lanceolate to narrowly ovate, 3-5 x 5-8 cm, both surface pubescent, margins serrate, apex mucronate or obtuse. Cauline leaves reduced, sessile, 3-lobed on inconspicuously sheaths. Peduncles slender 15-23 cm, pubescent; bracts 1-3, linear or lacking; rays 9-15, unequal, 3-7(-10) cm, pubescent; bracteols 2-5, linear or linear-lanceolate, umbellules 20-25-flowered. Calyxx teeth prominent, linear. Petals white, outer flowers of umbels radiant.

Fruit obovoid, 8-13 x 6-9 mm, glabrous when mature; dorsal ribs filliform, lateral ribs broadly winged, wings wider than body; vittae subclavate, solitary in each furrow, extending to 1/2 length of mericarp, 2 abbreviated ones or lacking on commissure, filliform, extending to 1/2 length of mericarp. Seed face plane. Fl. Jul.– Sept., fr. Aug.-Sep.

Distribution: East Nepal: 3000-3300 m [Bhutan].

4. *Tetrataenium sublineare* (C.B.Clarke) Mandenova ex Farille, Cauwet-Marc & Malla, Candollea 40(2): 562 (1985).
Heracleum sublineare C. B. Clarke, f. Fl. Brit. Ind. 2: 713. 1879; *Tetrataenium sublineare* (C.B.Clarke) Manden., Zametki Sist. Geogr. Rast. 41: 47 (1986).

Plants 20-50(-100) cm high. Root slender, cylindrical. Stem slender, solitary, slightly branched, pubescent. Basal leaves petiolate; leaf blade ovate, 10-15 x 8-11 cm, ternate-pinnate; leaflets sessile, mostly llinear or linear-lanceolate, sometime ovate, 3-10 x 0.4-0.8 cm, both surface pubescent, especially along veins, apex acute, margins serrate. Cauline leaves similar to basal, slightly sheathing, leaflets narrow simple pinnate. Umbells 3.5 - 7 cm across; bracts 1-3 or lacking, linear; rays 6-10, unequal, 1.5-3 cm, pubescent; bracteols 2-5, linear or linear-lanceolate, umbellules 8-14 flowered. Calyx teeth prominent, linear. Petals white, creamy pink, outer flowers of umbels radiant; radiant petals 2-lobed. Fruit oval to obicular, 5-7 x 4-6 mm, glabrous when mature; dorsal ribs filliform, marginal ribs narrowly winged, wings narrower than body; vittae filliform, solitary in each furrow, extending to 3/4 length of mericarp, 2 on commissure, filliform, extending to 3/4 length of mericarp. Seed face plane. Fl. and fr. Jul.-Oct.

Distribution: East Nepal: 3100-4000 m [Bhutan].

5. ***Tetrataenium lallii*** (C.Norman) Cauwet, J.Carbonnier & M.Farille, Candollea 37(2): 558 (1982).
Heracleum lallii C.Norman**,** in Journ. Bot. 1929, lxvii. 247.

Plants stout to 40-90 cm high. Stem erect, sparsely branched, pubescent throughout, covered with scaly remains of petioles at base. Basal leaves petiolate; leaf blade oblong or narrowly oblong. 16-25 x 5-12 cm, pinnate, pinnae 2-5 pairs; leaflets sessile or subsessile, ovate 2.5-6 x 2-6 cm, sparsely hairy on both side, margins irregularly crenate or serrate, often slightly lobed. Cauline leaves similar to the basal, moderately sheathing. Umbells 8-15 cm across; bracts 1 or lacking; rays 15-30, 3.5-7 cm, unequal; bracteols 2-3, lanceolate, umbellules 12-30 flowered.

Calyx teeth lanceolate- triangular or obsolete. Petals white, oval to obovate, the outer strongly radiant, unequally emaginate. Fruit narrowly oblong, 5-7 x 4-6 mm; dorsal ribs filliform, prominent; marginal ribs narrowly winged, wings narrower than body; vittae large, subclavate, 1-2 in each furrow, extending to base, 4 on commissure, filliform, extending to base. Seed face plane. Fl. and fr. Aug.-Sep.

Distribution: West, Central Nepal: 3000-4000 m.

6. *Tetrataenium nepalense* (D. Don) Mandenova. Trudy Tbilis. Bot. Inst., 20: 17 (1959).
Heracleum nepalense D. Don, Prodr. Fl. Nepal. 185. 1825; *Heracleum nepalense* var. *bivittatum* C.B. Clarke, Fl. Brit. Ind. 2: 714. 1879.

Plants stout, to 200 cm high. Root cylindrical. Stem solitary, pubescent. Basal leaves long petiolate; leaf blade broad ovate, 20-50 x 15-30 cm, ternate or 1-2-pinnate, pinnae 2-3 pairs; leaflets ovate to broadly ovate, entire to largely 3-lobed, 5-20 x 4-15 cm, both surface finely pubescent, apex obtuse or acute, margins coarsely serrate. Cauline leaves similar to the basal, reduced upward, inconspicuously sheathing, leaflets slightly to acutely 3-lobed. Peduncle stout, 10-15 cm. Umbells primary terminal umbels very large 20-35 cm across, distinctly over topped by the smaller secondary lateral umbels; bracts 1-2, linear or absent; rays numerous, 20-30(or more), 4-10 cm, extending in fruit; bracteols 3-5, linear, unequal; umbellule 20-35 flowered. Calyx teeth prominent, linear. Petals white or pinkish yellow , outer flowers in umbel radiant; radiant petals 2-lobed. Fruit elliptic or broadly obovate, 8-12x 5-10 mm; dorsal ribs prominent, low-keeled, filliform, marginal ribs broadly winged, wings wider than body; vittae subclavate or filiform, solitary in dorsal furrows, extending to 1/2-2/3 length of mericarp, 1-2 in lateral furrows, shorter than dorsal, 2-4 on commissure, about 2/3 as long as mericarp. Seed face plane. Fl. and fr, Jun.-Aug.

Distribution: West, Central, East Nepal: 1800-3700 m [NE India, Myanmar, Yunnan, Bhutan].

7. *Tetrataenium woodii* (M.F.Watson) Paik & M.F. Watson, stat. nov.
Heracleum woodii M.F. Watson, Edinburgh J. Bot. 53(1): 133. 1996.
Heracleum nepalense D.Don var *bivittatum* C.B.Clarke, Fl. Brit. Ind. 2: 714. 1879.

Plants stout, 50-100 cm high. Root stout, cylindrical. Stem solitary, pubescent. Basal leaves long petiolate; leaf blade broad ovate, 20-50 x 15-30 cm, pinnate, leaflets ovate, 3-5 ternately lobed or divided, 5-20 x 4-15 cm. Cauline leaves similar to the basal, slightly sheathing, leaflets

triangular-ovate, rounded, shallow to deeply lobed. Peduncles Umbells primary terminal umbels 3-6 cm across (to 10cm in fruit), held above the subtending solitary secondary lateral umbels, bracts 1-2, linear or absent; rays numerous, 10-15(-20), 3-5 cm, extending in fruit; bracteols 3-4, lanceolate, unequal; umbellule (12-)16-25-flowered. Calyx teeth prominent, triangular. Petals greenish-yellow or brownish yellow, rarely whitish. Fruit elliptic or broadly elliptic-obovate, 6-10x 6.5-8 mm; dorsal ribs prominent, low-keeled, filliform, marginal ribs broadly winged, wings equal or slightly narrower than body; vittae linear, solitary in dorsal furrows, extending to 2/3 length of mericarp, 1-2 in lateral furrows, shorter than dorsal, 2 on commissure, about 2/3 as long as mericarp. Seed face plane. Fl. and fr, Jul.-Sep.

Distribution: Central Nepal: 2000-4000 m [Bhutan].

Note: Similar to ***T. nepalense*** but a smaller less robust plant and generally less hairy.